Synthesis of cysteine sulfoxides and related compounds occurring in wild onions

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Dedicated to my beloved parents and wife for their precious supports and gentle, who have inspired me and been the driving force throughout my life.

Foreword

The majority of the genus *Allium* species are known for their medicinal applications: for example, *A. stipitatum* shows antibiotic effects against *Mycobacterium tuberculosis* and some other *Allium* species exhibit antistaphylococcal effects. Furthermore, recent studies have provided evidence of various antifungal, antidiabetic, and anticancer effects of some *Allium* species.

Allium is also well known in traditional medicine for its ability to cure various ailments such as wounds or acute and chronic bronchitis; additionally, alliums can be used as expectorants due to the presence of cysteine sulfoxide and its derivatives. Cysteine sulfoxides, which are some of the basic secondary compounds in *Allium*, have been addressed in various studies. Alliin, propiin, methin, marasmin, and thiosulfinate, all of which are alliinase substrates, are of particular interest because of their pharmaceutical effects. The enzymatic reaction that occurs during the formation of disulfides or thiosufinates results in various compounds that differ chemically, physically, and pharmaceutically.The cysteine sulfoxide recently investigated in *A. giganteum, A. rarolininanum, A. rosenorum,* and *A. macleani* species is the S-(pyrrolyl)cysteine S-oxide.

The formation of the red pigment after dissecting tissues in the *Allium* species indicates a quick reaction between cysteine sulfoxides and alliinase.
The natural acquisition of compounds from the *Allium* species and their in vitro storage are constrained by their high instability. Thus, any further studies on the molecular structure and mechanisms of S-(pyrrolyl)cysteine S-oxide required it to be synthesized chemically in laboratory settings. This would enable us to obtain the desired product (red pigment) through the laboratory reaction of the pyrrole compounds with alliinase.

To this end, we attempted to synthesize the heteroaromatic 5-ring compounds, which are homologous with S-(pyrrolyl)cysteine S-oxide, to understand the red pigment and its reaction mechanisms with alliinase.

Acknowledgements

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List of Abbreviations

TAPC	1,3,5-Triazo-2,4,6-triphosphorine-2,2,4,4,6,6-tetrachloride
ATP	Adenosine triphosphate
ACSO	Allylcysteinesulfoxide
ALA	Aminolevulinic acid
atm	Atmosphere
Cbz	Benzyloxycarbonyl
C-NMR	Carbon-13 Nuclear Magnetic Resonance
CSs	Cysteine sulfoxides
DNA	Deoxyribonucleic acid
DCM	Dichloromethan
DMF	Dimethyl formamid
DMSO	Dimethyl sulfoxide
ETS	External transcribed spacer
FAD	Flavin adenine dinucleotide
GC	Gas chromatography
GPs	Glutamyl peptides
HPLC	High Performance Liquid Chromatography
IR	Infrared
ITS	Internal transcribed spacer
IEF	Isoelectric focusing
LDA	Lithium diisopropylamide
MMPP	Magnesium monoperoxyphthalate hexahydrate
MS	Mass spectroscopy
MCSO	Methylcysteinsulfoxide
n-Buli	n-Buthyl lithium
NADH	Nicotinamide adenine dinucleotide
trnL-F	Non-coding chloroplast sequences
NMR	Nuclear Magnetic Resonance Spectroscopy
OAS	O-acetylserine
PBG	Porphobilinogen
PG	Protecting group
t-BuOk	Potasium-test-butanolat
PRN	Pro re nata
PCSO	Propylcysteinesulfoxide
PCSO	Propylcysteinesulfoxide
H-NMR	Proton Nuclear Magnetic Resonance
PXY-P	Pyridoxal-phosphate

rbcL	Ribulose bisphosphate carboxylase
RT	Room temperature
t-Buli	t-Buthyl lithium
BOC	Tert-butoxycarbonyl
THF	Tetrahydrofuran
TMEDA	Tetramethylethylendiamin
TLC	Thin-layer chromatography
TPCSO	Trans-propenylcysteinsulfoxide
TEA	Triethylamine

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1. General introduction

In recent decades, organic chemistry has progressed remarkably in biological and medical fields. Today, many medicines are made from organic substances to cure a wide range of indications. Among the living organisms, plants contain an abundance of organic compounds, which play a significant role in organic chemistry and biomedical processes. It is essential to understand the biological background and functionality of these biocompounds and their application in the medical fields. The biosynthesis of essential compounds of medicinally and economically important plant species has been the main focus of pharmaceutical research for years. Among the sulfuric plant compounds, cysteine sulfoxides play a significant role as precursors in *Allium* metabolism. These new natural compounds enable us to find a way to prevent various human diseases or in some cases postpone their occurrence. Therefore, it is important to investigate these bioactive compounds in genera such as *Allium*, the largest genus in the Amaryllidaceae family that has been studied largely for economic and medicinal reasons (Friesen et al., 2006).

1.1 The genus Allium: systematics, phylogeny, and distribution

The combination of plastid DNA sequences including *rbcL* and *trnL-F* with nuclear ITS showed Amaryllidaceae to be monophyletic with 48 genera and a sister group to asparagalean families (Meerow et al., 1999; Meerow et al., 2000). The genus *Allium*, likely one of the largest monocot's genera, consists of over 900 species (Fritsch & Abbasi, 2013; Seregin et al., 2015). It belongs to the order Asparagales, subfamily Allioideae, and family Alliaceae (Fay & Chase, 1996; Friesen et al., 2000; Chase et al., 2009; APG IV, 2016).

Linnaeus (1753) identified 30 species for the genus *Allium*. Taxonomy of the genus *Allium* is still controversial after the earliest monograph by Regel (1875). Vvedensky et al. (1935) identified nine sections and 228 species for the genus. Later, Traub (1968) introduced three subgenera including 36 sections with around 600 species in his revision. The species number and their taxonomic rank have been accordingly shuffled with the morphological, anatomical and karyological data (Kamelin, 1973; Stearn et al., 1980; Hanelt et al., 1990; Hanelt et al., 1992). Later, *Allium* was classified into 15 subgenera and 56 sections based on cytogeography, anatomy, and nuclear sequences (Nguyan et al., 2008). The most recent taxonomic classification of the genus was extensively modified according to the molecular phylogenetic study by Friesen

et al. (2006), which dramatically changed our understanding about species relationships and evolution. In general, the studies mainly based on ITS and ETS in the genus revealed three distinct evolutionarily lineages that contain 15 monophyletic subgeneric groups (**Figure 1.1**), among which some traditionally identified sections were raised to the subgeneric level, reaching 72 sections (Fritsch & Friesen, 2002; Friesen et al., 2006; Li et al., 2010; Choi et al., 2012; Mukherjee, 2013). Nevertheless, some groups still remain non-monophyletic (Friesen et al., 2006).

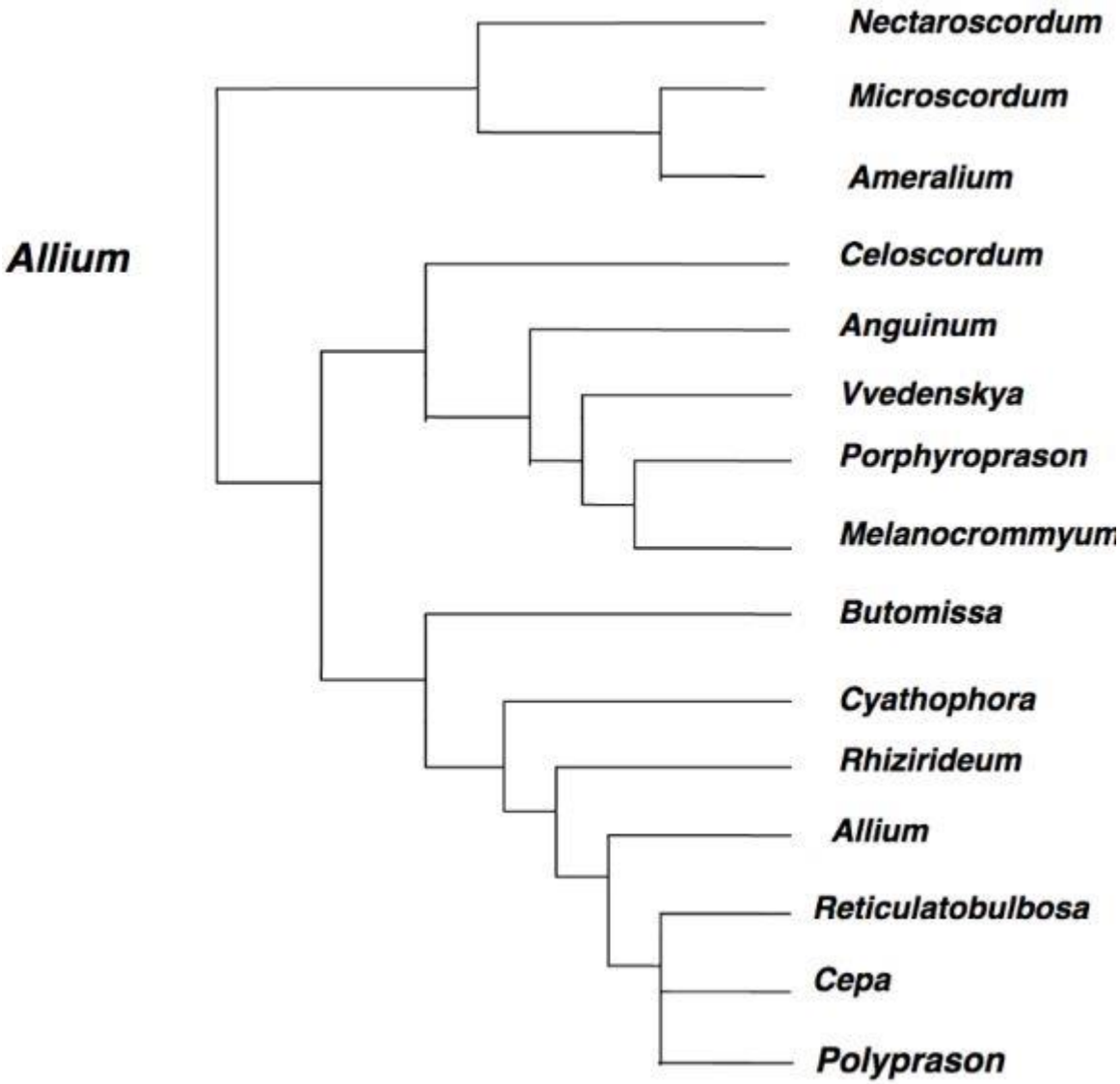


Figure 1.1 Infrageneric classification of the genus *Allium* (adopted from Friesen et al., 2006).

Morphologically, *Allium* species are perennial and characterized by their relatively globose to tunicate bulbs and mostly narrow basal leaves. The inflorescence is umbellate or head-like with usually straight to pendent flowers that are positioned on rigid scapes, and each flower usually

contains six free tepals, which form a free or basally connate calyx. Superior ovary includes one to many ovules in each locule and one entire or three-cleft stigma. The style is subgynobasic, capsules are loculicidal and seeds are rhomboidal black (Friesen et al., 2006; Fritsch et al., 2010). In general, leaf epidermal cell types (anticlinal), distribution, density, and type of stomata and trichomes are strong taxonomical traits for species delimitations (*e.g.* Ahmed et al., 2010). According to the latest studies, the genus *Allium* has a wide distribution (**Figure 1.2**) in seasonally dry areas with the highest diversity in Southwest, Central, and East Asia, Eastern Mediterranean, and North America (Fritsch & Friesen, 2002). The Irano-Turanian region has the highest concentration of endemic Allium species (Matin, 1992).

A narrow distribution has been reported for certain species, such as *A. schoenoprasum* from the subarctic belt, and some other species from Sri Lanka, Ethiopia, and Central America (Hanelt et al., 1990; Fritsch & Friesen, 2002). The occurrence of only one species, *A. dregeanum* in South Africa, is still debatable (Wilde-Duyfjes, 1976).

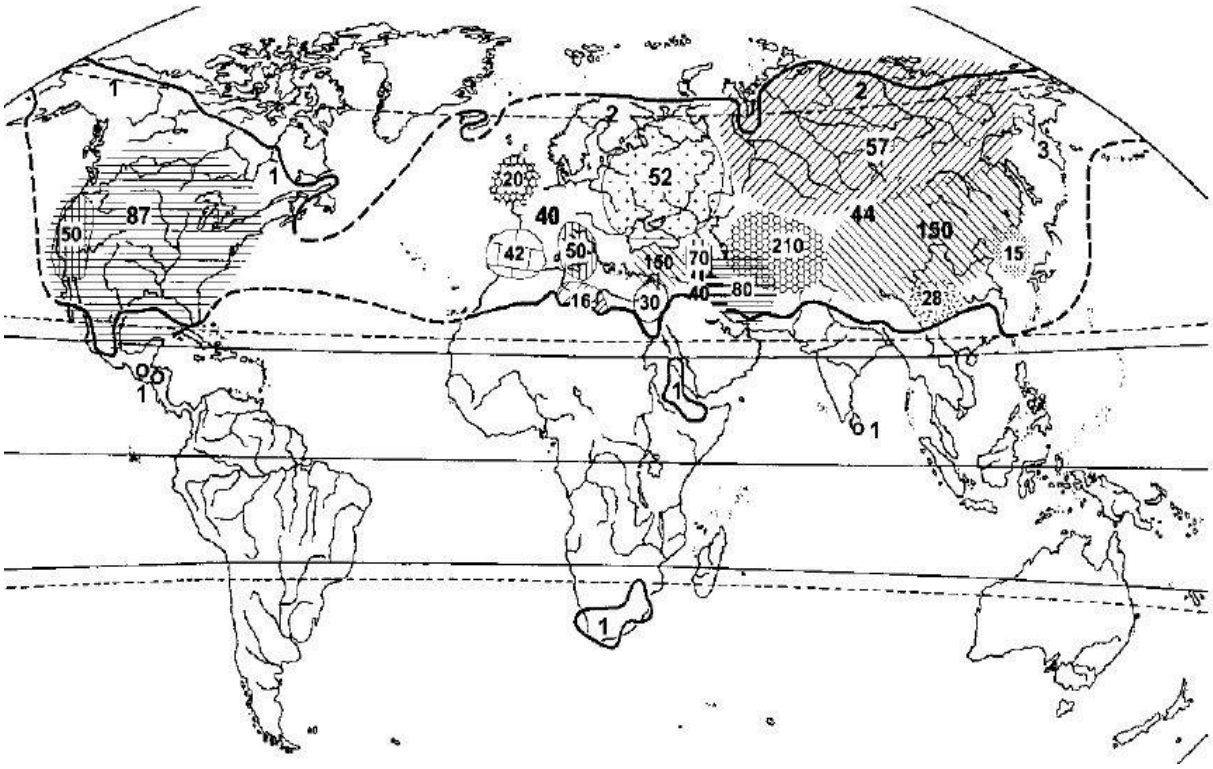


Figure 1.2 General worldwide distribution pattern of wild species of *Allium*. The numbers on the map correspond to the number of reported species in each region (Fritsch & Friesen, 2002).

Majority of *Allium* species are known for their vegetables and usage, and some others are economically important. Also, more than 100 species are ornamentals (Kamentsky and Fritsch, 2002).

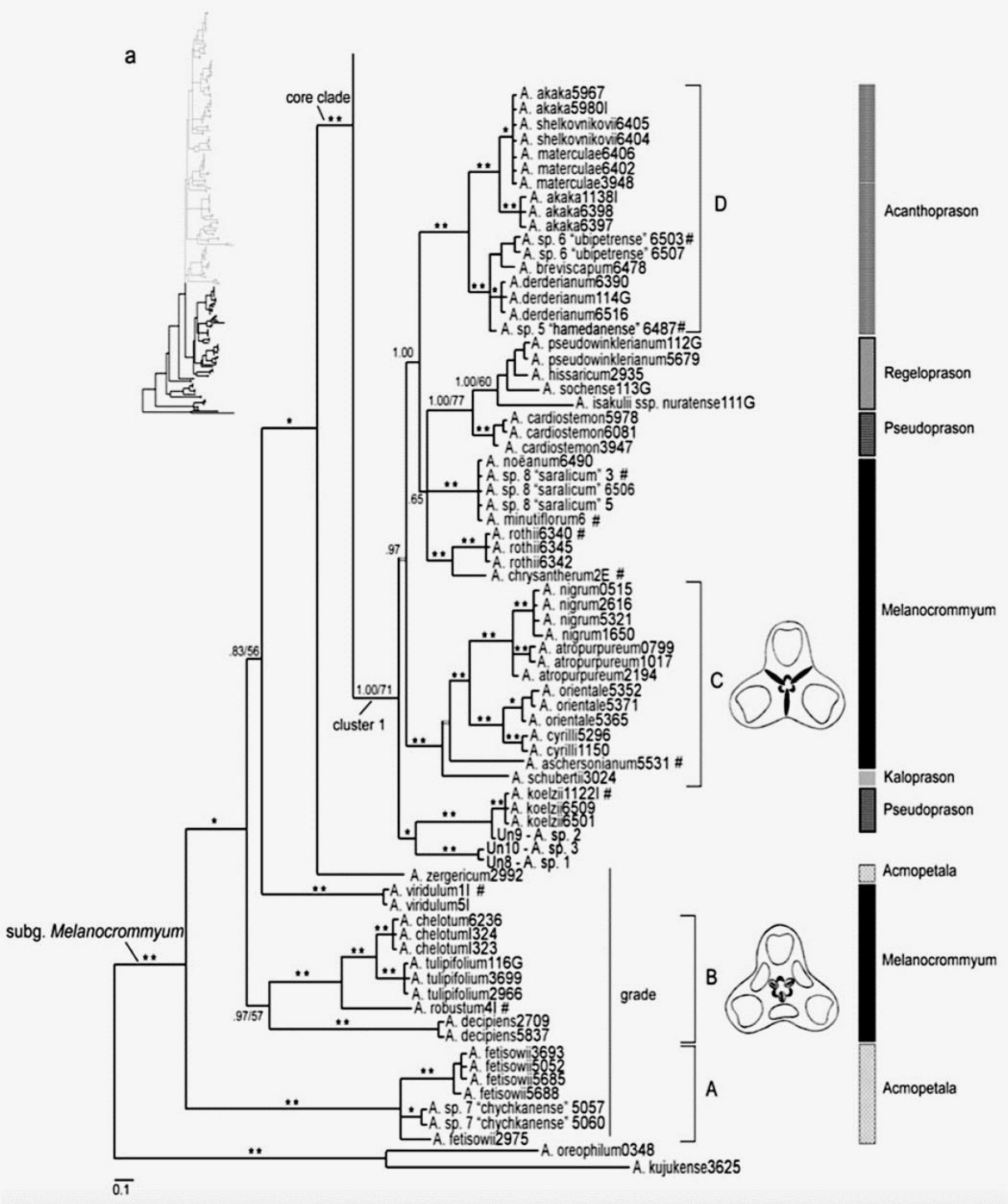


Figure 1.3 Phylogenetic relationships within the species of subgen. *Melanocrommyum* (adopted from Gurushidze et al., 2008).

1.2 A. subgen. Melanocrommyum taxonomy and phylogeny

Although most of the infrageneric groups are paraphyletic or polyphyletic according to the latest phylogenetic relationships based on ITS or plastid *trnL-trnF* sequences, the subgen. *Melanocrommyum* is determined to be monophyletic (**Figure 1.3**) (Gurushidze et al., 2008; Gurushidze et al., 2010).

Subgen. *Melanocrommyum* is one of the main subgenera of genus *Allium* comprising roughly 140 species (Khassanov & Fritsch, 1994; Fritsch et al., 2006). This number has increased to around 160 species that are mainly native to dry Mediterranean, Middle East, Northwestern China, and Central Asia (Fritsch et al., 2007; Seregin, 2007; Gurushidze et al., 2008; Fritsch et al., 2010).

Generally, it is mostly qualitative characteristics rather than the quantitative ones that have been used to delimit the species in the subgenus. According to previous studies, the most useful taxonomical traits related to qualitative characteristics include the shape of leaf blades, forms of tepals, scape length, shape of inflorescence, and flower density.

The reports referring to different numbers of infrageneric classifications in the genus, however, indicate the taxonomic uncertainties of species within the subgenus. Many of its species are known only for their ornamental flowers, and they are rarely economically important.

The best-studied species of *Allium*, such as *A. sativum*, *A. schoenoprasum*, *A. ursinum*, *A. cepa*, and *A. ameloprasum* var. *porrum* (Kucerová et al., 2011), have been used as medicinal plants for the treatment of various diseases. They contain steroidal saponins and various organic sulfur compounds, such as cysteine sulfoxides (Sobolewska et al., 2016). In this study, *A. sativum* and *A. giganteum* in subgen. *Melanochrommyum*, characterized by novel heteroaromatic cysteine sulfoxide compounds and metabolites, have been of particular interest (Jedelská et al., 2008; Kubec et al., 2011).

1.3 Important Allium species used in folk medicine

Allium contains many economically and medically important species such as onion, bunching onion, and chives, which are being used mostly as vegetables or spices (Fritsch & Friesen, 2002) by local residents (Fritsch & Khassanov, 2008). Medical advantages of *Allium* species are often referred to in relation to their antioxidant effects (*e.g*. Yin & Cheng, 1998; Tepe et al., 2005). Recently, antioxidant biocomposites have been generated from the red onion extract (de Dicastillo et al., 2015). Anti-diarrhea and anti-parasite effects of *Allium* date back to ancient Egypt (Keusgen et al., 2002). In Middle Asia and Europe, green *Alliums* has been largely used as a condiment, owing to its characteristic taste and smell. In the food industry, wild onion is mainly used as a spice, while in the pharmaceutical industry, it is a common ingredient in food supplements. In addition, the consumption of *Allium* species as a source of therapeutic benefits has a long history in common folklore (González et al., 2009). For centuries in ancient Greece, *A. sativum* (garlic), known for its therapeutic and medicinal effects in modern and traditional medicine, has been used to treat diseases such as animal bites, leprosy, plague, heart disorders, and cancer (Rose et al., 2005). Garlic has a long medicinal history in a fight against heart diseases because of its cholesterol-lowering, antibacterial, and antiartherosclerotic properties (Keusgen et al., 2002). In some areas of China, garlic is used as a folk medicine for the prevention of colds. Some species, such as *A. stipitatum* and *A. severtzovioides* of subgen. *Melanocrommyum*, are useful for curing stomach and duodenum disorders (Keusgen et al., 2006). The species such as *A. stipitatum* and *A. rosenbachianum* are often used as medicinal herbs for many ailments (Keusgen et al., 2006). *A. cepa* has antidiabetic (Bang et al., 2009) and antiasthmatic effects (Wagner et al., 1990). Garlic and onion are species rich in sulfur compounds, including mainly cysteine derivatives (S-alkyl cysteine sulfoxide). Volatile compounds such as thiosulfinate and polysulfinate have antidiabetic, antibiotic, blood sugar lowering, and other biological effects (El-Demerdash et al., 2005). Recent folk medicine usage of species such as *A. pskemense* from Uzbekistan and *A. saworowi* from Tajikistan illustrates their impact against tuberculosis (Keusgen et al., 2006). Moreover, *A. jodanthum* has been reported to cure blood fever and toothache, and *A. komarowii* is used to mitigate problems related to anemia, bronchitis, and tuberculosis (Keusgen et al., 2006). Also, *A. rosenorum* was found to have an inhibitory effect on epidermal staphylococcus (Jedelská et al., 2008), and *it* serves as a traditional body tonic (Keusgen et al., 2006) and food since its leaf is used as a spice in soups (the so-called atolla and oshi sioalaf). *A. carolinianum* is also used both as a vegetable

to cure swelling, dysentery, and arthritic problems, and as a body tonic (Khan & Khatoon, 2008).

1.4 Chemical compounds of genus Allium

1.4.1 Non-sulfur compounds of *Allium*

The most common non-sulfur compounds in alliums include carbohydrates, flavonoids, and saponins. Carbohydrates affect largely the flavor of the species, and flavonoids contain phenolic groups that exist, for example, as quercetin in *A. cepa*. Rodríguez Galdón et al. (2008) identified five different types of quercetins: isoquercetin, quercetin diglucoside, quercetin monoglucoside 1, quercetin monoglucoside 2, and free quercetin. In their research, more than 80% of the total quercetin was in the form of quercetin monoglucoside 1 and quercetin diglucoside. Also, Inoue (1995) found spirostanol and furostanol saponins in some *Allium* species.

1.4.2 Sulfur compounds of genus *Allium*

Sulfur is a key nutrient for plant growth (*e.g*. Buchner et al., 2004). Onion's sulfate present in roots converts into cysteine, methionine, or peptides such as glutathione in the leaves (Saito, 2004). The amino acid cysteine and its derivatives, the. S-substituted cysteine sulfoxides and the γ-glutamyl peptides, are the most common non-protein sulfur compounds in alliums (Block et al., 1993). Relevant gamma-glutamyls were identified in different CSs (Kasai & Larson, 1980). The first sulfur-containing volatile compound in alliums, the sulfonic acid, is produced when alliinase reacts with its substrate (CSs) after the cell dissection (Manabe et al., 1998). In addition, metabolites such as thiosulfinate, thiol, and disulfide are produced as secondary structures from sulfonic acid under non-enzymatic reaction (Block et al., 1993).

1.4.2.1 Cysteine sulfoxides (CSs)

Among the numerous compounds related to the genus *Allium*, the most interesting ones are those responsible for the volatile odor. These volatile compounds are formed by the hydrolyzation of non-volatile organic sulfur enzymatic compounds, the so-called S-alkenyl cysteine sulfoxides (CSs), the main secondary compounds in the cytoplasm of *Allium* species. To date, only positive secondary compounds of (+)-cysteine sulfoxides have been reported in *Allium* species (Krest et al., 2000).

Many of the economically important *Allium* species produce CSs (Fritsch & Keusgen, 2006) that have various medicinal effects (Block et al., 1993); their contents and chemical structures

vary for different species and usually correspond to taxonomic groupings (Fritsch & Keusgen, 2006). It seems that the relative amount of cysteine sulfoxides in *Allium* is species-specific (Keusgen et al., 2002). Also, it is supposed that S-substituted cysteine derivatives in alliaceous species are often used as storage compounds for sulfur or nitrogen (Kubec et al., 2011). For instance, S-(2-pyrrolyl) cysteine S-oxide's presence in the intact bulbs of *A. giganteum* was studied by Kubec et al. (2011). Additionally, it has been proven that (N-substituted-2-pyrrolyl) cysteine S-oxides have possible antifungal and antibiotic activities.

The findings of Krest et al. (1997) indicate that different amounts of alliinase can determine the presence and estimate the amount of cysteine sulfoxides that are described further in the text. According to some research, different odors in different *Allium* species, such as onion and garlic, can be due to varying methods of storage of CSs or their different substrates resulting from the reaction of alliinase with CSs (Nock and Mazelis, 1987; Jansen et al., 1989; Fujita et al., 1990).

1.4.2.2 Different type of cysteine sulfoxides

Although many *Allium* species contain one or two cysteine sulfoxides (Krest et al., 2000; Fritsch & Keusgen, 2006), *A. tuberosum* contains more (Manabe et al., 1998). **Figure 1.4** shows the most famous types of cysteine sulfoxides in tribe Alliaceae including S-(2-pyrrolyl)cysteine-S-oxide, (+)-S-(ethyl)-L-cysteine sulfoxide or ethiin, (+)-S-(methylthiomethyl)-L-cysteine sulfoxide or marasmin, (+)-S-(butyl)-L-cysteine sulfoxide or butiin, (+)-S-(benzyl)-L-cysteine sulfoxide or petiveriin A, (+)-S-(benzyl)-L-cysteine sulfoxide or petiveriin B, (+)-S-(3-pentenyl)-L-cysteine sulfoxide, (+)-S-(2-hydroxyethyl) cysteine sulfoxide, and (+)-S-(3-pyrrolyl)-L-cysteine sulfoxide (Kubec et al., 2000; Kubec and Musah, 2001; Kubec et al., 2002; Kubec et al., 2002b; Kubec et al., 2013; Dini et al., 2008; Jedelská et al., 2008).

The four main basic compounds of cysteine sulfoxides are: (i) alliin or (+)-S-C2-(2-propenyl)-L-cysteine sulfoxide, present in high amount in garlic; (ii) methiin (MCSO) or (+)-S-methyl-L-cysteine sulfoxide, present in low amount in garlic; (iii) isoalliin (TPCSO) or S-(1-propenyl)-L-cysteine sulfoxide; and (iv) propiin (PCSO) or (+)-S-propyl-L-cysteine sulfoxide in onions and leeks (Virtanen & Matikkala, 1959; Granroth, 1970; Freeman & Whenham, 1975; Lancaster & Kelly, 1983; Block et al., 1993; Breu, 1996; Fritsch & Keusgen, 2006).

S-allyl-cysteine sulfoxide (ACSO) or alliin was the first precursor compound identified *in vitro* by Stroll & Seebrook (1947). Alliin attaches to pyridoxal-phosphate (pxy-p) and converts to S-alk(en)yl-cysteine sulfoxide and volatile compounds in *Allium*. Alliin is one of the non-volatile thiosulfinate compounds responsible for the flavor of *A. sativum* (Block et al., 1993; Hornickova et al., 2010).

S-Methyl-L-cysteine sulfoxide (methiin) is the most ubiquitous compound that shows in varying amounts in the intact tissues of all *Allium* species, mainly in *A. sativum, A. cepa, A. porrum, and A. urisinum*.

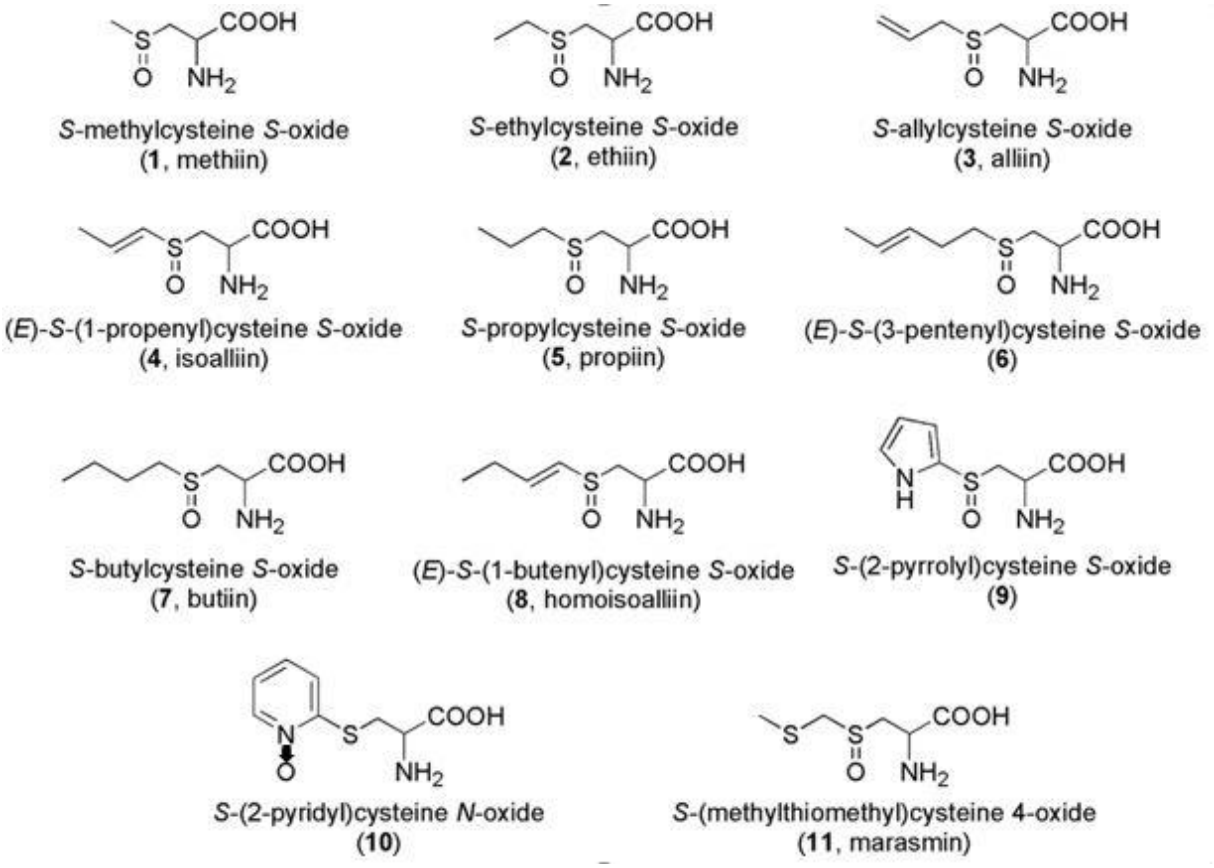


Figure 1.4 Different types of cysteine sulfoxides in tribe Alliaceae (adopted from Kubec et al., 2013).

The amounts or CS products resulting from analysis by alliinase significantly impact the severity of taste (Keusgen, 2013). Isoalliin, a source of the lachrymatory factor reference, is the major sulfoxide existing in intact "onion-type" tissues including *A. schoenoprasum* and *A. x proliferum* (top onion) (Fritsch and Keusgen, 2006). The formation of stable primary aroma compounds, such as thiosulfinates or the lachrymatory factor, is mediated by alliinase through the hydrolysis of S-alk(en)yl cysteine sulfoxides as a precursor (Rose et al., 2005) (**Figure 1.5**).

From these precursor compounds, through the action of alliinase, large amounts of sulfur compounds are synthesized. Additionally, *A. sativum* contains a significant amount of alliin, and isolliin is abundant in *A. cepa* (Koch & Lawson, 1996).

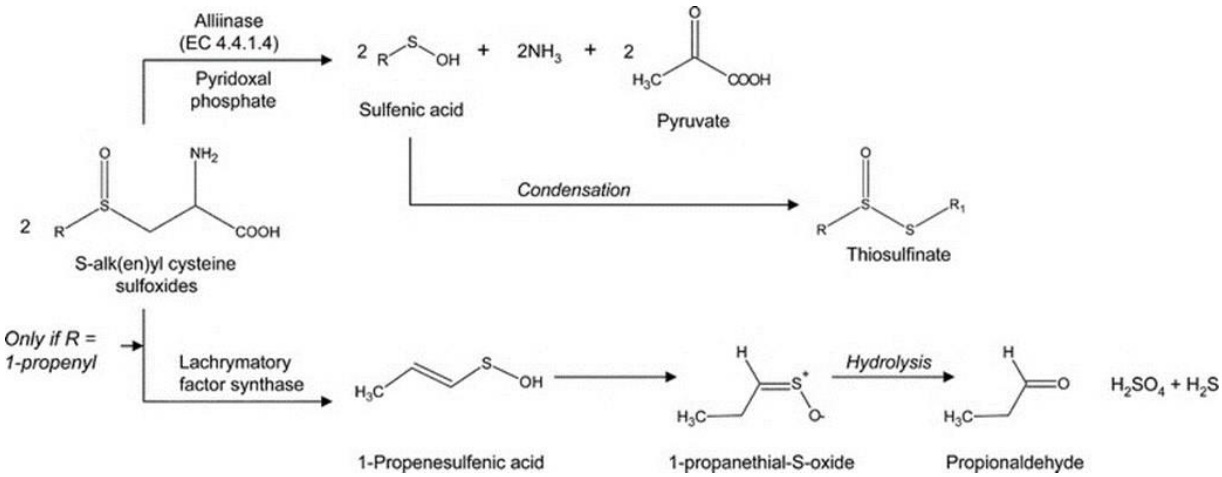


Figure 1.5 Hydrolysis of S-alk(en)yl cysteine sulfoxides mediated by alliinase results in the formation of sulfenic acids, which produces thiosulfinates or the lachrymatory compound 1-propanethial-S-oxide (Rose et al., 2005).

Allicin is present in *A. sativum* and lowers blood cholesterol (Lu et al., 2012). Marasmin, containing two sulfur atoms in each molecule, is produced after reaction by alliinase and is one of the classical cysteine sulfoxides that is often found in *A. stipitatum* and *A. suworowii* (Kusterer et al., 2011; Kubec et al., 2011).

1.5 Alliinase and biosynthesis of cysteine sulfoxides

1.5.1 Alliinase

Alliinase (EC 4.4.1.4) found in *Allium* species, commonly belongs to lyase enzymes and in particular to carbon-sulfur lyases. Being sensitive to pH and temperature, alliinase shows the best activity in *Allium* in C-S-lyzing TPCSO in comparison with MCSO or PCSO (cf. Nock & Mazelis, 1987). The first described alliinase was found in garlic (Nock and Mazelis, 1987; Fujita et al., 1990). Krest et al. (2000) found that in all studied *Allium* species, alliinase is activated in a pH range 5–8 and at an optimal temperature range 34°C–40°C, and is species-specific. In garlic, there are two different types of alliinase: one C-S-lyzes PCSO and TPCSO in pH = 4.5, and the other specific for MCSO action in pH = 6.5.

Jansen et al. (1989) indicated that metal cations including Fe^{+2} or Zn^{+2} as well as other cofactors such as ATP, FAD, and NADH, were not significantly correlated to alliinase activity or modifications. They also found that alliinase activity increases only in the presence of 10%

glycerol and pyridoxal phosphate. Kuettner et al. (2002) identified the structure of alliinase after Stroll and Seebeck (1947) first detected its action in *A. sativum*. It is assumed that alliinase likely exists in all species of *Allium* (Tsuno, 1958a; Lancaster, 2000). Its activity is commonly studied in *A. cepa*, *A. sativum*, *A. ampeloprasum*, *A. porrum*, *A. tuberosum*, *A. ursinum,* and *A. fitulosum* (Fujita et al., 1990).

Numerous studies have demonstrated that volatile sulfur compounds are responsible for the taste and flavor of *Allium* species. This is mainly caused by enzymatic hydrolyzing mediated by alliinase alk (en) yl-thiosulfinat S-alk (en) yl-2-cysteine sulfoxide (Block et al., 1993; Sendl et al., 1995; Koch & Lawson, 1996). The findings of Lancaster and Collin (1981) show that when an onion's tissue is damaged, the alliinase from vacuole breaks the CSs from the cytosol into sulfur and alpha carbon bonds. As **Figure 1.6** illsutrates, alliinase catalyzes the conversion of amino acid alliin ((+)S-allylcysteine sulfoxide) to allicin (diallyl thiosulfinate), pyruvate, and ammonia by utilizing pyridoxal-5′-phosphate (PLP) as a cofactor (Lancaster & Collin, 1981; Weiner et al., 2009). Alliinase breaks the C-S bond in the reaction of CSs and pyridoxal-5′-phosphate by means of beta-elimination using a shiff base (Breu, 1996). Alliinase has specificity for alliin and isoalliin (Nock & Mazelis, 1987; Rabinkov et al., 1995; Krest et al., 2000). In addition, using isoelectric focusing (IEF) method, Nock and Mazelis (1987) have separated isoenzymes in alliinase that are chemically and physically different from each other.

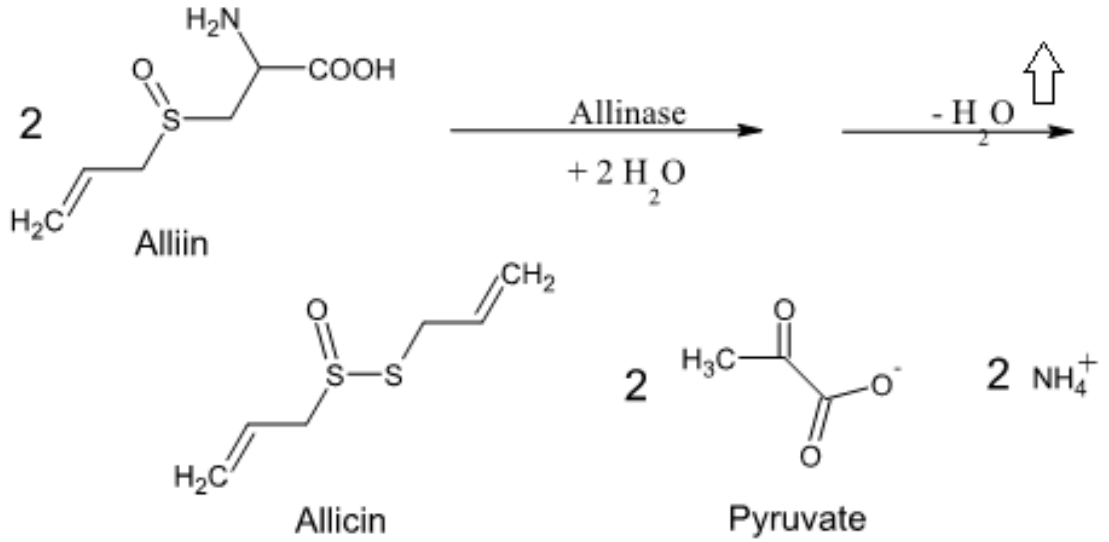


Figure 1.6 Reaction of alliinase on alliin (after Weiner et al., 2009).

In addition to *Allium*, some other species such as *Petiveria alliacea* (Musah et al., 2009), *Albizia lophanta* (Schwimmer & Kjær, 1960), broccoli, and other Brassicaceae, (*e.g*. Hall & Smith, 1983) contain alliinase with a significantly different structure from that of the *Allium* species (Hamamoto & Mazelis, 1986). Moreover, alliinase and its relevant compounds are also found in some bacteria (Reily, 1980; Kamitani et al., 1990; Chhabria & Krutika, 2016) and fungi (Iwami and Yasumoto, 1980).

Ellmore and Feldberg (1994) showed that aliinase is placed in sheath cells also available in these cells .They also found that the existence of alliinase in epidermic cells of onion, confirmed by auto-fluorescence, may act as an antimicrobial defense through stomata. According to some other studies, the type of alliinase and its activity reaction to cysteine sulfoxides differs in the bulb and root of the onion (Ramírez and Whitaker, 1998; Lancaster et al. 2000). Also, different isozymes of alliinase found in *Allium* differ not only physically and chemically, but also in terms of their enzymatic activity (Nock & Mazelis, 1987). Additionally, being composed of 1470 base pairs, genome sequences of allinase, with a circular DNA shape, have been identified in some *Allium* species using cloning approaches (Van Damme et al., 1993; Clark et al., 1993). Alliinase activity can be inhibited by cysteine sulfoxide derivations, carboxyl methyl amine, and hydroxyl amine (Jansen et al., 1989).

Alliinase, for instance in an allium's bulb, is active as a monomer that reacts with cysteine sulfoxide and catalyzes the reaction. It also links the amine group of cysteine sulfoxides to aldimine and subsequently makes electron emission to break cysteine sulfoxide into sulfur-carbon. Breaking aldimine activates cofactor-enzyme and forms α- aminoacrylic acid through pyridoxal-5′-phosphate (Krest et al., 2000). Following this reaction and after cleavage of cysteine sulfoxides, sulfuric and α-aminoacrylic acids are formed as primary products followed by primary flavor compounds (Krest et al., 2000).

The effect of alliinase on different cysteine sulfoxides results in various secondary compounds. For instance, different compounds including methyl propyl trisulfide, di-2-propenyl disulfide, methyl-2-propyl-disulfide, and methyl-1-propyl-disulfid are produced in *A. ursinum* (Schmitt et al., 2005). The other types of mono-, di-, and trisulfides in *A. ursinum* are responsible for its odor and flavor (Keusgen, 2013). The red pigment is also produced as the result of the cleavage reaction by alliinase. The occurrence of red pigments in some alliums, for example in *A. giganteum* (subgen. *Melanochrommyum*) is due to the enzymatic reaction accumulating 2-lactyl-3′-pyrrolyl sulfoxide to 3,3′-dithio-2,2′-dipyrrole. Consequently, L-(+)-S-(3-

pyrrolyl)cysteine sulfoxide is first degraded and then natural red pigments are formed in *A. macleani* after bulb disruption (Jedelská et al., 2008). These types of cysteine sulfoxides, consisting of two structures, can be polymerized to dark red under oxidative reaction (Keusgen, 2011).

1.5.1.1 Effect of pH on enzymatic activity and substrate stability

Jansen et al. (1989) used the optimum pH of 6.7–7 to estimate the alliinase enzymatic activity extracted from *A sativum*. Nevertheless, previous studies reveal that the optimum pH depends on the species from which the enzyme is obtained. For example, the pH of 7 is determined to be optimum for *A. altyncolicum* (Keugen et al., 2002) and *A. fistulasum* (Fujita et al., 1990). In different species of subgen. *Melanocrommyum,* for example *A. macleanii,* two optimum pHs were identified due to the existence of two subunits or isoforms of alliinase, representing different activities in pH = 7.5 and pH = 5 (Vogt, 2008-doctoral thesis). The maximum activity of *A. macleanii* was reported in the pH = 10 (Vogt, 2008-doctoral thesis). The pyrrolyl cysteine sulfoxides existing in subgen. *Melanocrommyum* are similar to the substrate used in this test. Also, the enzyme alliinase extracted from *A. stipitatum* contains two different optimum pHs, pH = 7.7 and pH = 8.2, while *A. rosenorum* has only the optimum pH = 7.7 (Mielke, 2015-doctoral thesis).

1.5.1.2 Effect of temperature on enzyme activity and substrate stability

Reactivity temperatures for the alliinse extracted from *A. sativum, A. altyncolicum,* and *A. stipitatum* are 33°C (Jansen et al., 1989), 36°C (Keusgen et al., 2002), and 36°C (Mielke, 2015-doctoral thesis), respectively. Generally, the optimum temperature of alliinase for different species of *Allium* varies between 30–40°C (Keusgen et al., 2002). This optimum temperature reported for *A. rosenorum* is 39°C (Mielke, 2010) and for *A. macleanii* 36°C (Vogt, 2008-doctoral thesis). These two species contain pyrrole cysteine sulfoxide compounds and are considered important.

1.5.2 Biosynthesis pathway of cysteine sulfoxides

Chemically unstable metabolites of CSs make it relatively difficult to analyze them and their pathways. Nowadays, we appreciate various methods and techniques such as HPLC or GC for direct analysis of different CSs or their derivatization in plant tissues (Rose et al., 2005) and it is very common to use various synthetic methods including HPLC, NMR, IR, and photometry to find biosynthetic pathways (kusterer et al., 2011).

Cysteine is a water-soluble amino acid required for the biosynthetic pathway in the plant species. Moreover, direct chemical investigation and radiolabel feeding help us understand the biosynthesis pathway of CSs. Radiolabeled carbon and sulfur sources show that cysteine sulfoxides are rapidly incorporated into the glutamyl peptides (GPs) (Rose et al., 2005). All types of CSs have a primary source of sulfur (SO_4^{2-}) to be synthesized (Hesse et al., 2004). Later, the synthesized SO_4^{2-} is converted into 5-adenylysulfate (APS) in a reaction catalyzed by the enzyme ATP sulfurylase in the plastids. Next, the APS is converted into sulfide through the enzyme sulfide reductase. Sulfide reacts with *O*-acetlyserine and forms cysteine utilizing the enzyme OAS thiol-lyase (Leustek et al., 2000). So far, nearly 24 sulfur-containing glutamyl peptides (GPs) have been identified in *Allium* species playing an intermediate role in CSs biosynthesis (Hesse et al., 2004).

Further studies show that γ-glutamylcysteine and glutathione are essential start-up compounds for CSs biosynthesis. Removing the glutamyl group by oxidation results in parental CSs (Rose et al., 2005). Granroth (1970) proposed an alternative pathway, in which cysteine reacts with methacrylic acid and forms S-2-carboxyproplyl cysteine, and S-2-carboxypropyl glutathione (containing radiolabel ^{14}C) results in ACSO, TPCSO and PCSO. In another study, Randle and Lancaster (2002) introduced a biosynthesis pathway for different peptides and S-alk(en)yl cysteine sulfoxides (ACSOs) in *Allium*. Additionally, according to Lancaster and Show (1989), gamma-glutamyl-cysteine sulfoxides intermediate the synthesis of CSs.

1.5.2.1 Biosynthesis pathway of known cysteine sulfoxides

Since the 1940s, many studies have been conducted to understand the biogenesis pathways of compounds like methiin, alliin, and propiin, although they remained relatively poorly understood.

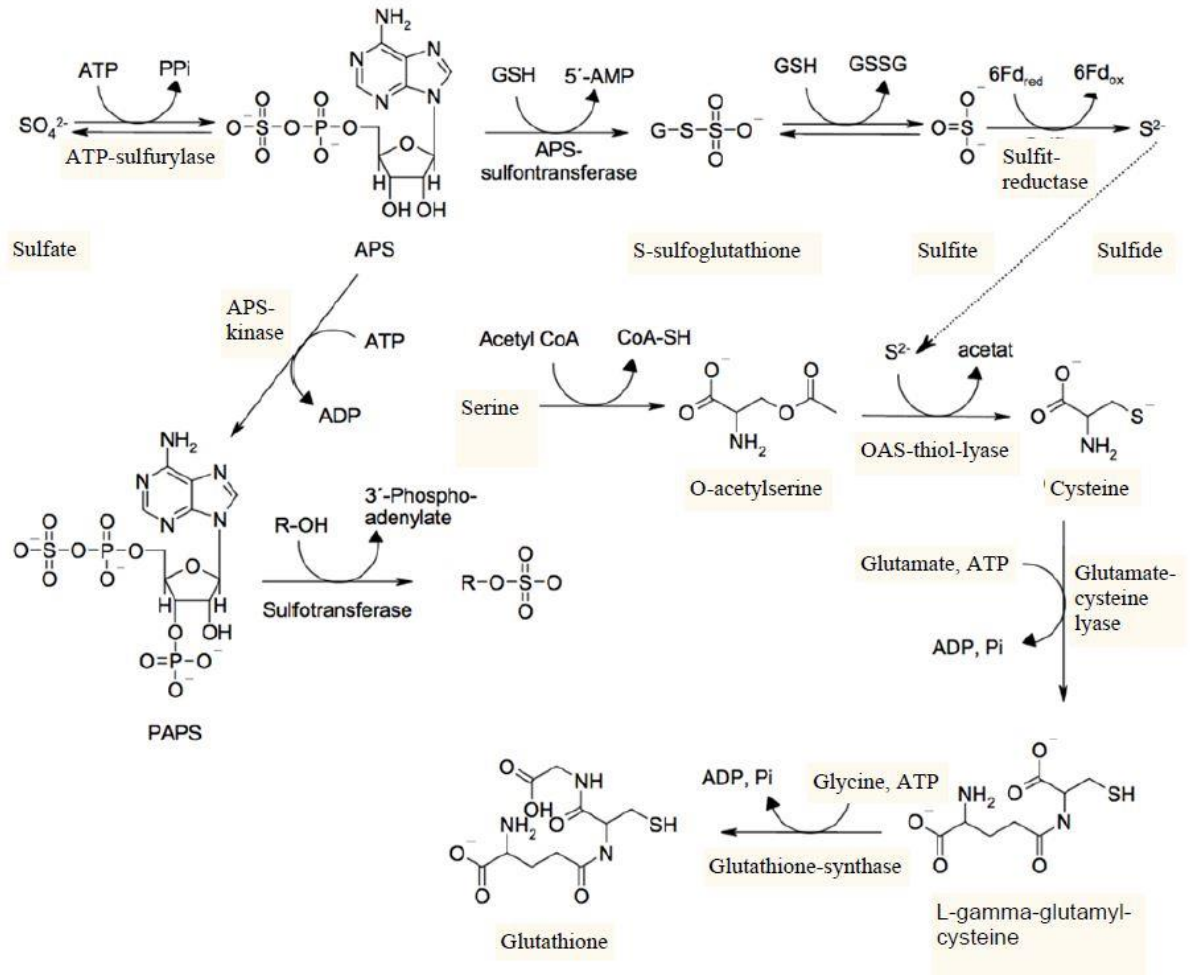


Figure 1.7 Assimilation of sulfate in plants (after Luestek and Saito, 1999).

However, Rose et al. (2005) identified many compounds together with the related enzymes incorporated in their biogenesis pathway including gamma-glutamyl peptide. The gamma-glutamyl peptide compound, as a storage for sulfur and nitrogen, plays a key role in the formation of cysteine sulfoxide (Rose et al., 2005).

Lancaster and Show (1989) proposed one of the most accepted theories regarding the formation of cysteine and glutathione in the plants. They also described the biosynthetic pathway required for the formation of methiin, propiin, and isoalliin from glutathione (**Figure 1.7** and **Figure 1.8**). Moreover, it is estimated that nearly one-third of the formed cysteine is immediately transferred to glutathione's cycle (Jones et al., 2004).

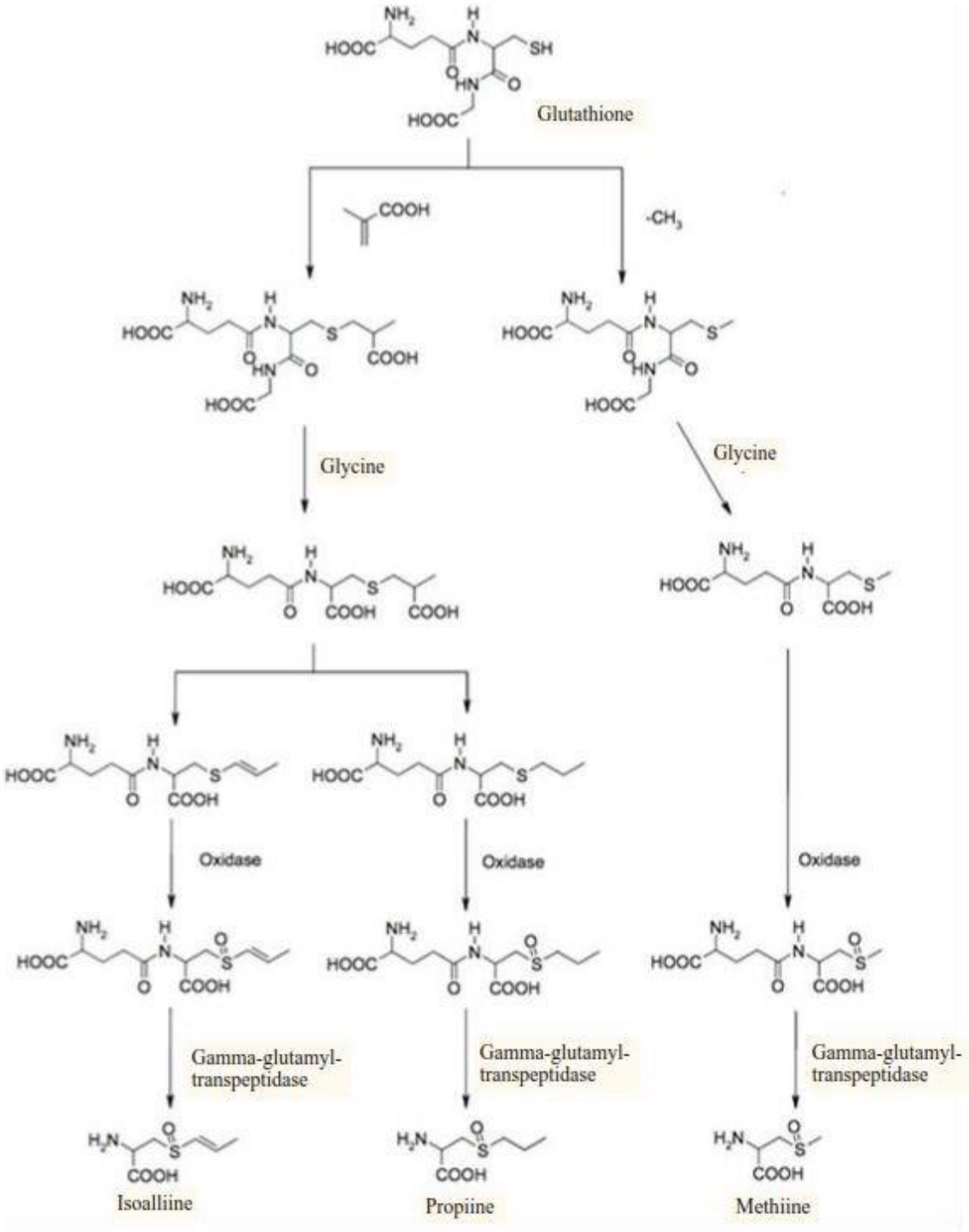


Figure 1.8 Proposed biosynthetic pathway of isoalliine, methiine, and propiine (after Lancaster and Show, 1989).

1.5.3 Biosynthesis pathway of pyrrolyl cysteine sulfoxide

As is the case for other cysteine sulfoxides, no biosynthetic pathway has been suggested for (pyrrolyl)cysteine sulfoxide compound. This compound is available in subgen. *Melanocrommyum* species such as *A. macleani* (Jedelská et al., 2008), *A. rosenorum* (Jedelská

et al., 2008), *A. giganteum* (Kubec et al., 2011), and *A. carolinianum*. Also, glutathione compounds and precursor substances play an important role in the biosynthesis of this new compound (Vogt, 2008-doctoral thesis). In order to present a biosynthesis model for the (pyrrolyl)cysteine sulfoxide compound, it is necessary to first describe some of its related primary compounds.

1.5.3.1 Prodigiosin

Prodigiosin is one of the important pyrrole compounds (**Figure 1.9**) in plants, and its biosynthesis from aminolevulinic acid (ALA) was described by Williamson et al. (2006).

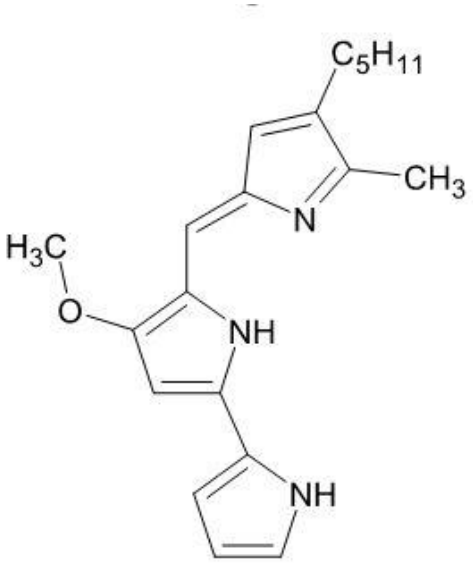


Figure 1.9 Molecular structure of prodigiosin.

1.5.3.2 L-tryptophan

Tryptophan, an essential amino acid, is one of pyrrole compounds in plants (**Figure 1.10**).

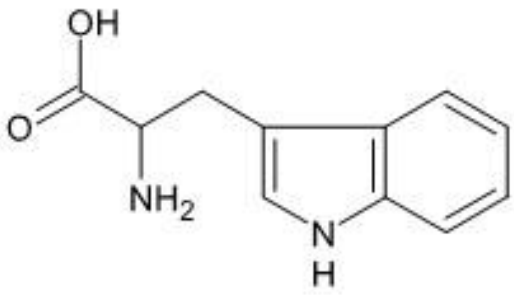


Figure 1.10 Molecular structure of L-tryptophan.

Figure 1.11 illustrates the formation of amino acid L-tryptophan from n-(5'-phosphoribosyl) anthranilate and L-serine (Crawford, 1989).

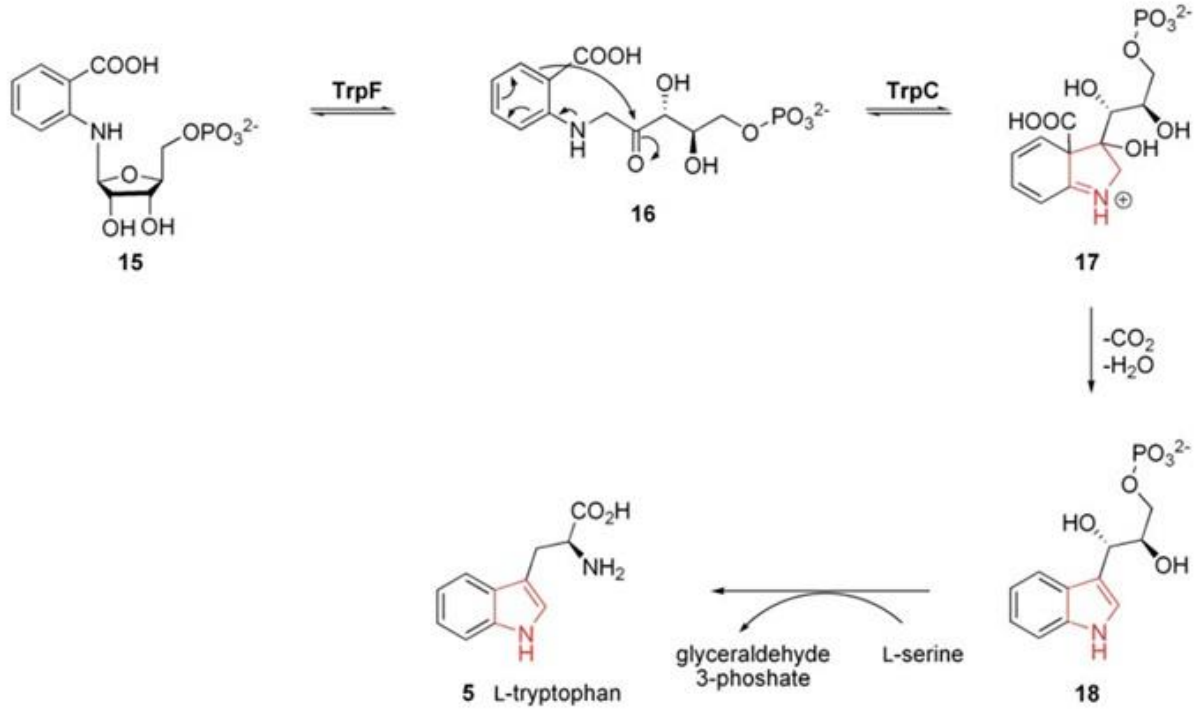


Figure 1.11 Biosynthesis of tryptophan from N-(5phosphoribosyl)anthranilate (adopted from Crawford, 1989).

1.5.3.3 Porphobilinogen

Although porphobilinogen (PBG) (**Figure 1.12**) does not have any effect on the abovementioned biosynthetic pathway in plants, it is one of the significant compounds containing pyrrole. It is worth noting that the amino acid glycine and acetate are both involved in the formation of PGB.

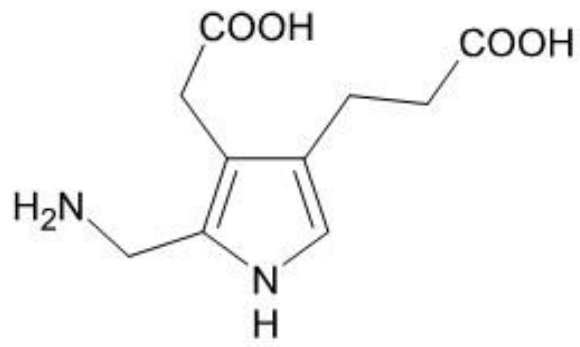


Figure 1.12 Molecular structure of porphobilinogen.

Acetate is transferred to the citrate cycle and converted into succinyl-CoA, which is converted into ALA following decarboxylation of glycine. This substrate is the primary substrate for formation of PGB (Lehninger, 1987), (**Figure 1.13**).

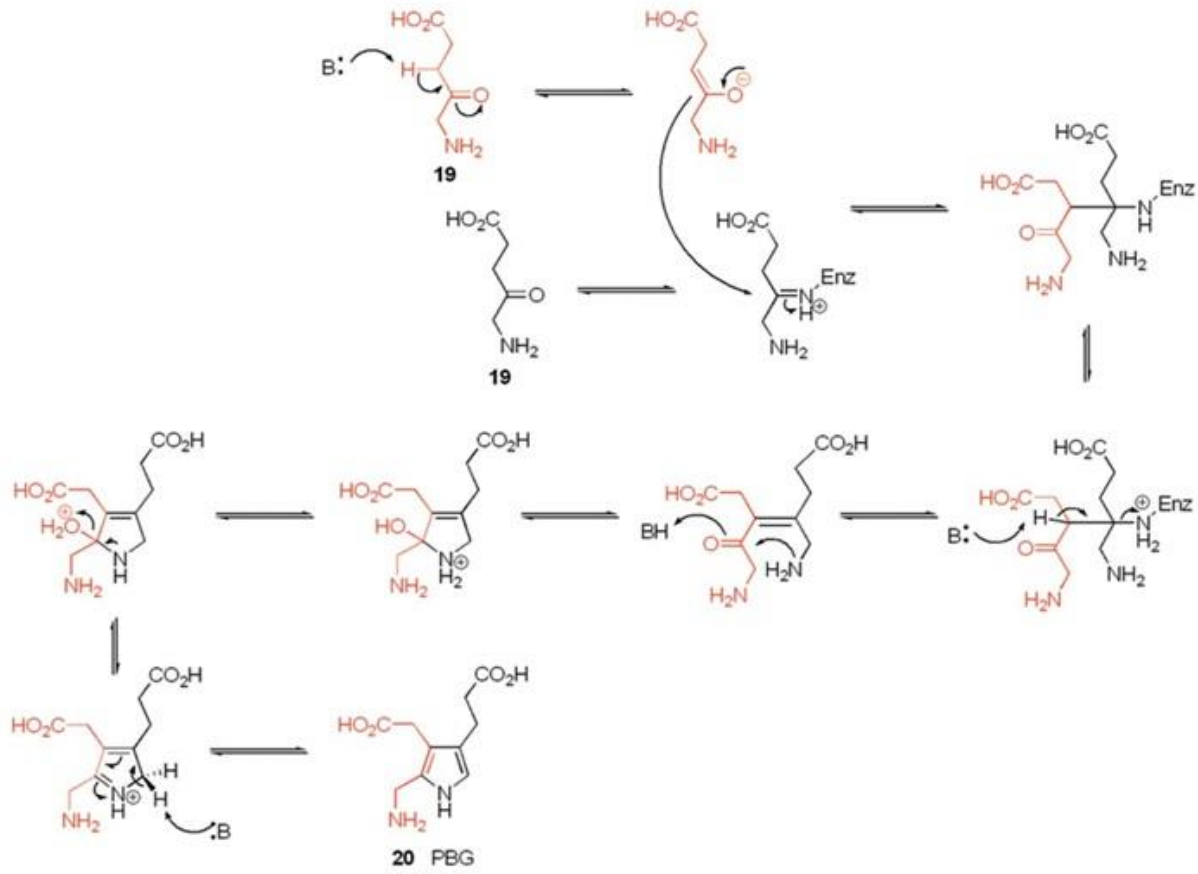


Figure 1.13 Biosynthesis of porphobilinogen and formation by ALA dehydratase (Jordan, 1991).

1.5.3.4 Pyrrole-2-carboxylate

The enzymatic oxidation of proline during a complex reaction (**Figure 1.14**) results in the formation of pyrrole-2-carboxylate that is one of the precursors for many pyrrole compounds in plants (Williamson et al., 2006).

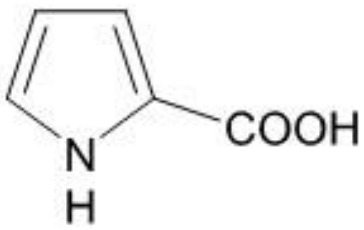


Figure 1.14 Molecular structure of pyrrole-2-carboxylate.

1.5.3.5 Methylpyrrole-2,4 dicarboxylic acid

3-Methylpyrrole-2,4 dicarboxylic acid (**Figure 1.15**) as a central pyrrole moiety is one of the essential compounds in the synthesis of coumermycin A1, an amino coumarin antibiotic consisting of *Streptomyces rishiriensis* and containing 3-pyrrole rings with 5-methylpyrrole-2-carboxylomoieties at terminals, and 3-methylpyrrole-2,4 dicarboxylic acid moiety at the center (Siebenberg et al., 2011).

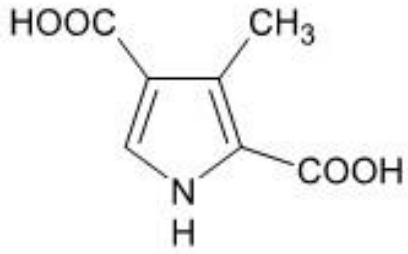


Figure 1.15 Molecular structure of 3-methylpyrrole-2,4 dicarboxylic acid.

1.6 Amino acids involved in the synthesis of cysteine sulfoxides

1.6.1 Cysteine

Berg et al. (2002) found that non-essential amino acids such as serine, cysteine, and alanine, can be converted into pyruvate. Boyle (2005) showed that among 20 amino acids existing in protein structures, aliphatic non-polar alanine contains CH_3 side chain, while uncharged polar amino acids, including cysteine and serine, contain CH_2SH and CH_2OH side chains, respectively. In addition, the sulfur atom, one of the essential elements of cysteine, plays a significant role in plant growth. Cysteine is also an important source of sulfide in human metabolism and reduces the toxic effect of alcohol. In plant metabolism, biosynthesis of cysteine is one of the key components in the sulfur cycle. Plant's roots absorb sulfate ions from the soil and and these ions enter the biosynthesis of cysteine (Singh, 1998). Cysteine is then produced in cytosol, plastids, and mitochondria through the enzymes serine acetyl transferase and o-acetylserine(thiol)lyase (Álvarez et al., 2012). Later, the produced cysteine is incorporated in protein structure and glutathione as sulfur in methionine or secondary sulfuric compounds, such as allyl cysteine sulfoxide (Singh, 1998). Additionally, cysteine is considered as a primary precursor for essential biomolecules like vitamins, cofactors, antioxidants, and many protective compounds in plants.

1.6.2 Serine

Serine is naturally available as L-isomer in proteins. L-serine belongs to the non-essential amino acids that can be synthesized in the human body under normal physiological circumstances. Serine can affect the structure of proteins. The hydroxyl group of its side chain enables serine to link the compounds via hydrogen bonds (Betts & Russell, 2003). Being the precursor to several amino acids including glycine and cysteine, it participates in the biosynthesis of purines and pyrimidines during metabolism activities. It also serves a significant role in maintaining the immune system (Betts and Russell, 2003). L-serine, whose isoelectric point is at pH 5.68, belongs to non-essential amino acids synthesized under normal physiological circumstances in human body (Betts and Russell, 2003). It is fully soluble in water, but only slightly soluble in ethanol and diethyl ether.

1.6.3 Alanine

Alanine, a non-essential α-amino acid, appears naturally as an L-stereo isomer in proteins that can be produced by the human body. It is distinguished by its α-carbon linked to a methylene group and is likely the dullest amino acid that is considered neither hydrophobic nor polar. Alanine is rarely directly involved in the protein function. However, alanine shows substrate specificity and interacts with non-reactive atoms like carbons (Betts & Russell, 2003).

1.7 Research scope and objectives

The reaction of alliinase with heteroaromatic cysteine sulfoxides like S-(2-pyrrolyl)cysteine-S-oxide and S-(3-pyrrolyl)cysteine-S-oxide produces the remarkable red pigment. These cysteine sulfoxides can be found in *A. carolinianum*, *A. giganteum*, *A. macleanii*, and *A. rosenorum*, where convert into the red pigment after the cell dissection. The homologous compounds were synthesized like furan and thiophene cysteine sulfoxides were synthesiszed , which are usefull for comparing their reactivity, synthesis pathways, and structures. Additionally, an alliinase bioactivity test was conducted on both sulfoxide and sulfide compounds in order to identify the resulting products. Thus, the study aimed to address the following goals: (i) the suitable synthetic pathway of pyrrole cysteine sulfoxides and homologous heteroaromatic compounds (**Figure 1.16**); (ii) possible proposed structures of these compounds (**Figure 1.17**); and (iii) the hypothesized biosynthetic pathway of pyrrolyl cysteine sulfoxide.

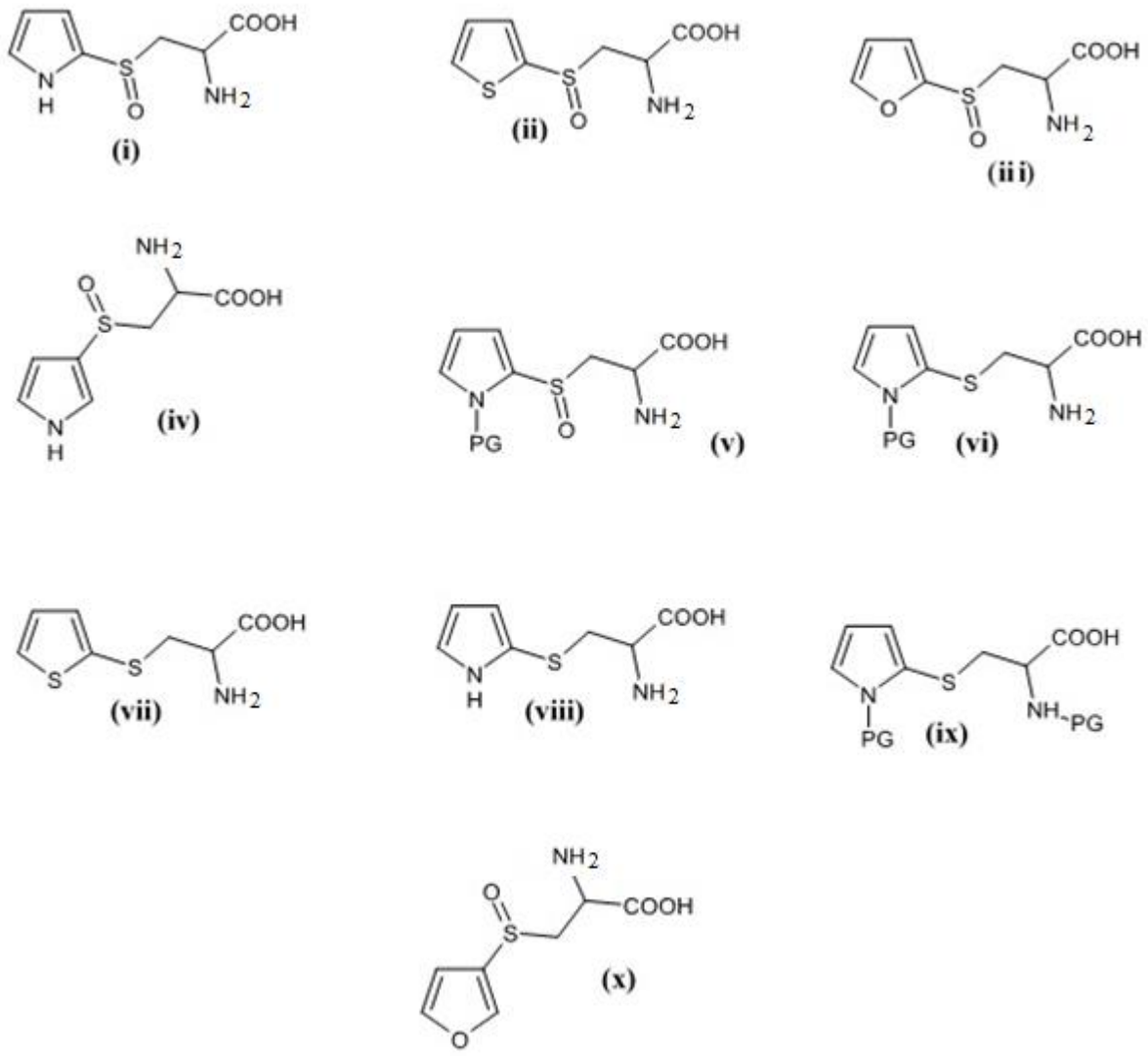


Figure 1.16 The structures of the compounds aimed to be synthesized. (i) S-(2-pyrrolyl)cysteine-S-oxide; (ii) S-(2-thienyl)cysteine-S-oxide; (iii) S-(2-furanyl)cysteine-S-oxide; (iv) S-(3-pyrrolyl)cysteine-S-oxide; (v) S-(N-(PG)pyrrol-2-yl)cysteine-S-oxide; (vi) S-(N-(PG)pyrrol-2-yl)cysteine; and (vii) S-(2-thienyl)cysteine; (viii) S-(2-pyrrolyl)cysteine; (ix) protected pyrrolyl cysteine; (x) S-(3-furanyl)cysteine-S-oxide. PG can be Me, ph, Boc, or Cbz.

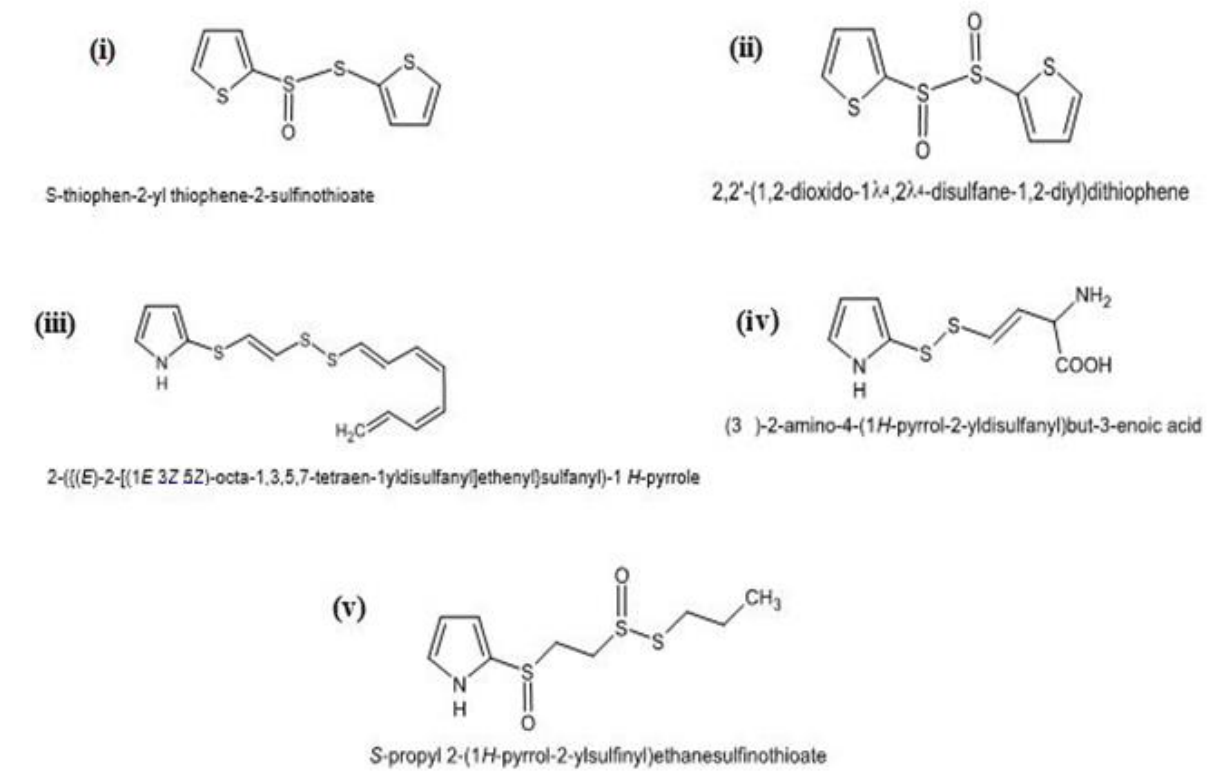


Figure 1.17 Molecular structures of (i S-(2-thienyl)thiophene-2-sulfinothioate; (ii) 2,2´-(1,2-dioxido-1 λ4, 2 λ4-disulfane-1,2-diyl)dithiophene;(iii)2-({(*E*)-2-[(1*E*,3*Z*,5*Z*)-octa-1,3,5,7-tetraen-1yldisulfanyl]ethenyl}sulfanyl)-1 *H*-pyrrole; (iv) (3*E*)-2-amino-4-(1*H*-pyrrol-2-yldisulfanyl)but-3-enoic acid and; (v) S-propyl 2-(1*H*-pyrrol-2-ylsulfinyl)ethanesulfinothioate

2 Methods and materials

Stock solvents and reagents were mainly obtained from Sigma-Aldrich (St. Louis, USA or Carl Roth, Karlsruhe Germany). The list of chemicals and reagents, and details of the devices are provided in **Appendixes 1–2**. Amounts of chemical reagents used for each experiment are listed in **Appendix 3**. All synthetic methods described here were developed in our lab. Thin layer chromatography (TLC), as one of the common approaches, was implemented to separate different compounds from the mixture. TLC was conducted on a silica gel plate (0.2 mm, silica gel 60) at F_{254} with a fluorescence indicator (Macherey Nagel, Dueren, Germany). The spots were detected using UV light under λ_{max} = 254 nm (*n*-Butanol:H_2O:acetic acid:formic acid, 28:8:9:2 *v/v/v/v*) for cysteine sulfoxide (after Keusgen, 1997; **Appendixes 2–3**). Ninhydrin reagent (Keusgen, 1999; **Appendix 3**) was used to recognize amino acids by TLC. Preliminary analysis of TLC was often further confirmed by supplementary analyses including ^{1}H-NMR,^{13}C-NMR, mass spectrometry (MS), infrared spectrometer (IR, Bruker, Ettlingen, Germany) analyses, and HR-MS unless otherwise mentioned. Structure elucidation was performed by various techniques, namely NMR, MS-ESI, MS-EI, and MS-HR. The 400 MHz NMR spectrometry was conducted with ECX-400 (Jeol, München, Germany) under the condition represented in **Appendix 2**. NMR spectra (^{1}H-NMR and ^{13}C-NMR) of isolated compounds were measured in D_2O or CD_3OD at 25°C. IR spectroscopy (Bruker alpha-P, software Opus 6.5) was used for characterization of functional groups and compounds. The synthetic methods for the main products in addition to the complementary approaches are presented in the following sections.

2.1 Synthesis of 3-chloro-L-alanine hydrochloride or β-chloro-L-alanine

L-Serine (2.5 g, 238 mmol), as a starting compound, was added to 250 ml tetrahydrofuran (THF). Hydrogen chloride was produced through the reaction of sulfuric acid (20–30 ml) with NH_4Cl (10–20 g) passing through the solution. The mixture was stirred at room temperature for 24 h until it was saturated with the gas. Thionyl chloride (6.25 g, 52.5 mmol) was slowly mixed into the solution. The reaction mixture was stirred at 50˚C for 3–4 days, concentrated (at 40˚C, 350 mbar) to about half the original volume, and cooled down to precipitate at 0–10˚C for 24 h. Eventually, the residue was washed several times with THF (10–20 ml), dissolved in methanol (5–10 ml), re-precipitated using diethylether (50–70 ml), and filtrated (after Yamashita et al.,

2003). Finally, it was analyzed using TLC, ^{1}H-NMR, ^{13}C-NMR, MS, HR-MS, and IR. The synthesis is represented as in **Figure 2.1**.

The synthesized amount was estimated to be 21.52 g ≈ 0.204 mol.

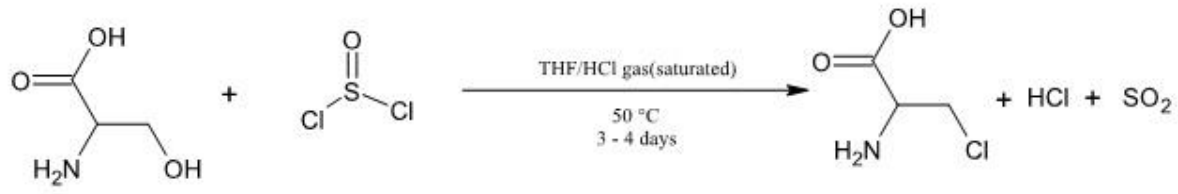


Figure 2.1 Synthesis of 3-chloro-L-alanine hydrochloride.

2.2 Synthesis of aliphatic cysteine sulfoxides

Aliphatic cysteine sulfoxides were synthesized according to Keusgen (1999): sodium (9.7 g, 420 mmol) was dissolved in 500 ml dry ethanol; next, L-cysteine (26.6 g, 200 mmol) was added to the mixture. Alk(en)yl bromides (220 mmol) were added to the reaction. The reaction's pH was set to 5.5 using acetic acid when the reagent cooled down at RT until full precipitation. The solution was stored overnight at 4°C to crystalize. The desired crystal (130 mmol) was dissolved in water (180 ml) after filtration. It was oxidized with H_2O_2 25% (15.2 ml, 130 mmol) to convert sulfide to sulfoxide. The solvent was removed under reduced pressure at 40˚C and 70 mbar. This method was used to synthesize S-(2-thienyl)cysteine in this study.

2.3 Synthesis of aromatic cysteine sulfoxide

2.3.1 Synthesis of S-(N-benzylpyrrol-2-yl)cysteine

N-Benzylpyrrole (3.17 ml, 20 mmol), thiourea (3.05 g, 40 mmol), 13 ml ethanol and 5 ml water were mixed at 30–40°C (mixture A). Potassium iodide (3.32 g, 20 mmol) and iodine (5.08 g, 20 mmol) were dissolved in 9 ml ethanol and 6 ml water (mixture B). Mixture B was gradually mixed with A and heated up to 30–40˚C for 3 h under argon protection. Two steps followed: i) hydrazine hydrate 35% (1 ml, 20 mmol) was added to the mixture of sodium hydroxide (3.00 g, 75 mmol) and 7 ml distilled water; ii) 3-chloro-L-alanine hydrochloride (3.84 g, 24 mmol) was dissolved in 8 ml distilled water. The mixtures obtained in these two steps were added to the previous mixture of A and B. The resulting reaction mixture was heated up to 90–100˚C for 2 h, purified using activated charcoal (1–2 g) for 30–60 min., filtrated, and its pH was adjusted

to 5.5 by adding HCl 10% to precipitate the product. The obtained mixture was stored at 5˚C for 12 h. The product was preliminarily dried using rotary evaporator (at 40˚C, 70 mbar and 175 mbar for H_2O and ethanol, respectively) and further dried in the desiccator (containing P_2O_5) by applying the vacuum. Then it was dissolved in 10 ml methanol and centrifuged at 5,000 rpm at 4˚C for 10 min. The supernatant was precipitated with diethylether (50–100 ml) and stored at 4˚C for 24 h. Subsequently, the product was analyzed using TLC, ^{1}H-NMR, ^{13}C-NMR, MS, HR-MS, and IR (after Rudyakova et al., 2008). The synthesized amount was estimated to be 2.58 g ≈ 9.3 mmol.

2.3.2 Synthesis of S-(N-methylpyrrol-2-yl)cysteine

The mixture of N-methylpyrrole (2.7 ml, 30 mmol), thiourea (4.56 g, 60 mmol), 15 ml ethanol and 8 ml water were heated up to 30–40˚C (mixture A). Potassium iodide (4.98 g, 30 mmol) and iodine (7.61g, 30 mmol) were dissolved in 16 ml 50% alcohol/water (1:1 *v/v*) (mixture B). Mixtures A and B were gradually passed through argon and heated up to 30–40˚C for 3 h. Hydrazine hydrate 35% (1 ml, 20 mmol) was mixed with sodium hydroxide (4.78 g, 75 mmol) in 8 ml distilled water, and added to the mixture produced in the former step. 3-Chloro-L-alanine hydrochloride (5.77 g, 36 mmol) was dissolved in 15 ml distilled water, added to the reaction mixture from the previous step and heated up to 90–100˚C for 2 h. The product was purified using activated charcoal (1–2 g) for 30–60 min., filtrated, and the pH was reduced to 5.5 using HCl 10% to obtain precipitation. The mixture was stored at 5˚C for 12 h under argon protection. The resulting product was preliminarily dried using rotary evaporator (at RT, 40 mbar and 110 mbar for H_2O and ethanol, respectively) and further dried in the desiccator (containing P_2O_5) through the vacuum. It was then dissolved in methanol (10 ml) and centrifuged at 5,000 rpm at 4°C for 10 min. Re-precipitation was conducted using diethylether (50–100 ml) and the residue was stored at 4˚C for 24 h. Finally, it was analyzed using TLC, ^{1}H-NMR, ^{13}C-NMR, MS, HR-MS, and IR (after Rudyakova et al., 2008). The synthesized amount was estimated to be 1.35 g ≈ 6.75 mmol.

2.3.3 Synthesis of S-(2-pyrrolyl)cysteine

The mixture containing pyrrole (1.42 ml, 20 mmol) in addition to thiourea (3.05 g, 40 mmol) plus 13 ml ethanol and 5 ml water was heated up to 30–40˚C. Potassium iodide (3.32 g, 20 mmol) and iodine (5.07 g, 20 mmol) were dissolved in ethanol/water (9:6 *v/v*). The two reagents were gradually mixed under argon at 30–40˚C for 3 h. Hydrazine hydrate 35% in H_2O (1 ml, 20 mmol) was mixed with sodium hydroxide (3.00 g, 75 mmol) in 7 ml distilled water. All chemicals mentioned above were added to the mixture resulted from the former step. 3-Chloro-L-alanine hydrochloride (3.84 g, 24 mmol) was dissolved in 8 ml distilled water, added to the previous one, and heated up to 90–100˚C for 2 h. The product was purified using activated charcoal (1–2 g) for 30–60 min, filtrated, and the pH was reduced to 5.5 using HCl 10%. The resulted precipitation in the mixture was stored at 5˚C for 12 h. The end product was preliminarily dried using rotary evaporator (at RT, 40 mbar and 110 mbar for H_2O and ethanol, respectively) under argon protection and further dried in the desiccator (containing P_2O_5) through vacuum. Afterwards, it was dissolved in 10 ml methanol and centrifuged at 5,000 rpm at 4˚C for 10 min. Re-precipitation of the methanolic solution was conducted using diethylether (50–100 ml) and followed by 24-hour storage at 4˚C. The reaction was analyzed using TLC, ^{1}H-NMR, ^{13}C-NMR, MS, HR-MS, and IR (after Rudyakova et al., 2008). The synthesis is shown in **Figure 2.2**. The synthesized amount of the product was estimated to be 1.30 g ≈ 7 mmol.

2.3.4 Synthesis of S-(N-Bocpyrrol-2-yl)cysteine

N-Boc-pyrrole (3.4 ml, 20 mmol) and thiourea (3.05 g, 40 mmol) were added to 7.5 ml ethanol and 2.5 ml water while stirring. When thiourea was fully dissolved, the mixture of potassium iodide (3.32 g, 20 mmol) and iodine (5.07 g, 20 mmol) was added dropwise to the 10 ml ethanol/water solution (1:1 *v/v*). The temperature was controlled at 37°C under argon shield. After 3 h, hydrazine hydrate 35% (1 ml, 20 mmol) and sodium hydroxide (3.00 g, 75 mmol) were dissolved in 3 ml water and added to the solution while the temperature was maintained at 100°C. Next, 3-chloro-L-alanine (3.84 g, 24 mmol) was dissolved in 2.5 ml distilled water and the mixture was added to the reaction. The reaction lasted 2 h at 100°C. The resulting solution was purified using activated charcoal (1–2 g) for 30 min. To precipitate, HCl 10% was added to set the pH of the solution to 5.5. Then the liquid was removed by centrifugation (at 5,000 rpm at 4˚C for 10 min.) and consequently the residue dissolved in 10 ml methanol. Re-precipitation was implemented using diethylether (50–100 ml), centrifuged, and the mixture

was subjected to ^{1}H-NMR analysis (after Rudyakova et al., 2008). The synthesis is shown in **Figure 2.2**.

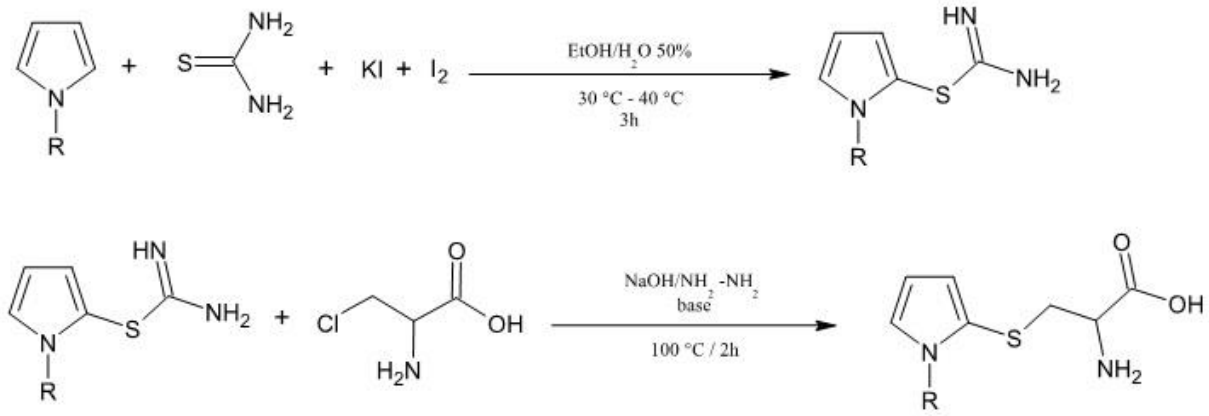


Figure 2.2 Synthesis of S-(N-Bocpyrrol-2-yl)cysteine. "R" in the structure of compounds indicates H, Me, ph, and Boc.

2.3.5 Synthesis of S-(2-thienyl)cysteine

The reaction conditions were derived from the synthesis of the aliphatic S-alkenyl-l-cysteine (Keusgen, 1999). Argon was used as the protecting gas. Due to the poor solubility of 3-chloro-L-alanine, the reaction mixture was heated up to 100˚C. In a three-necked flask, 20 ml pure ethanol was dried using sodium (386 mg, 1.68 mmol). Potassium hydroxide (561 mg, 10 mmol) was dissolved in the solution under argon protection before 2-thiophenethiol (2 mmol, 0.19 ml) was added. 3-Chloro-L-alanine (320 mg, 2 mmol) was mixed with the reagent and heated up to 100˚C for 2 h. The solution was filtered, its pH set on 5.5 with HCL 10%, evaporated (at 4˚C, 175 mbar) to reach 1 ml of total volume, and precipitated using 10 ml acetone. To obtain crystals, the precipitation was washed using ethanol and stored at 4˚C for 24 h. Consequently, the reaction was analyzed and visualized using TLC, ^{1}H-NMR, ^{13}C-NMR, MS, HR-MS, and IR. The synthesis is represented in **Figure 2.3**.

The synthesized amount was estimated to be 378 mg ≈ 1.86 mmol.

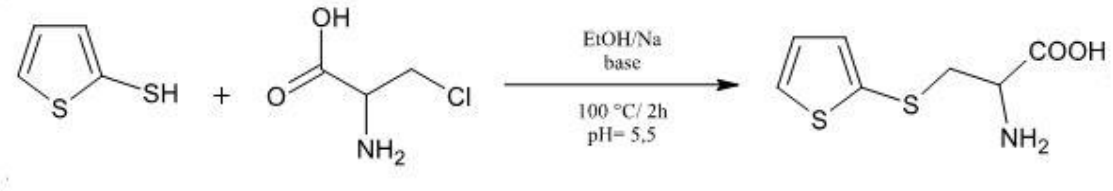


Figure 2.3 Synthesis of S-(2-thienyl)cysteine.

2.3.6 Synthesis of S-(3-furanyl)cysteine

In a three-necked flask, 2.5 ml ethanol was dried with sodium (48 mg, 2 mmol). L-Cysteine (120 mg, 1 mmol) was dissolved, 3-bromofuran (0.1 ml, 1.1 mmol) was added dropwise and the reaction stored under cold conditions for 12 h. The pH was reduced to 5.5 with HCL 10% and washed using ethanol after filtration. ^{1}H-NMR analysis was conducted on the reaction mixture (after Keusgen, 1999). The synthesis is shown in **Figure 2.4**.

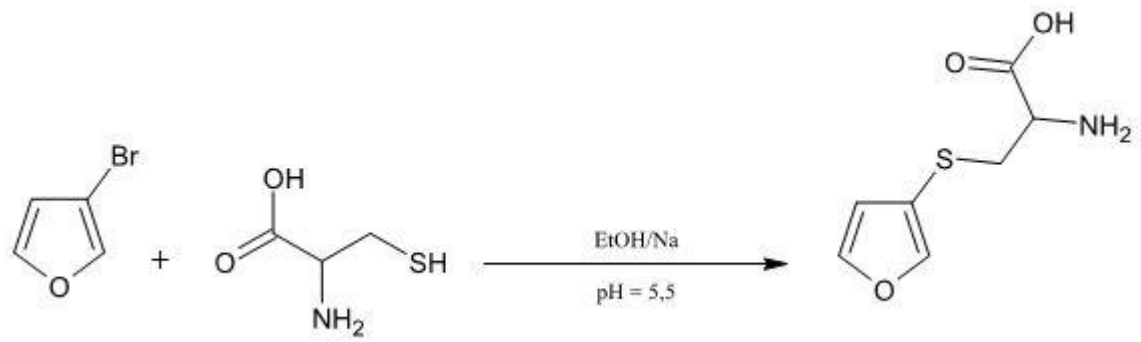


Figure 2.4 Synthesis of S-(3-furanyl)cysteine.

2.3.7 Synthesis of 1*H*-pyrrole-sulfonyl chloride

Chlorosulfonic acid (6.7 ml, 100 mmol) was carefully added dropwise to a mixture of pyrrole (3.5 ml, 50 mmol) and 12.5 ml chloroform ($CHCl_3$) at –10°C (as the reaction is exothermal reaction). The reaction was stirred at RT for 20 min. The yielded product was placed on ice, filtrated, and weighed to give a yield of 4.17 g. The reaction mixture was subjected to ^{1}H-NMR analysis. The synthesis is shown in **Figure 2.5** (Organikum, 2001, page 364).

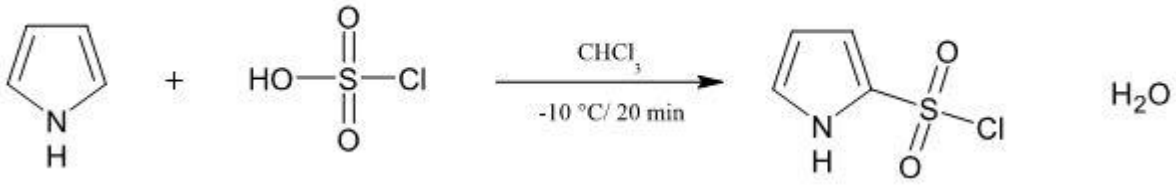


Figure 2.5 Synthesis of 1-H-pyrrole-sulfonyl chloride.

2.3.8 Synthesis of furan-3-sulfonyl chloride

Lithium was replaced with bromide in this reaction. 3-Bromofuran (0.9 ml, 1 mmol) was stirred in 20 ml THF at –78°C. t-Buli (0.19ml, 2 mmol) was added dropwise to the reaction over 10 min. The mixture was stirred at –78°C over 3 h. An electrophile ($ClHSO_3$, 0.07 ml, 1 mmol) was added dropwise and stored overnight at RT. The following steps were carried out under argon protection: 5 ml pentane and an equal amount of saturated sodium chloride were mixed together; the water phase was separated using 5 ml pentane; then, the organic phase was washed with 5 ml KOH 70% plus 5 ml saturated NaCl. It was further dried with $MgSO_4$ overnight at RT. The reaction mixture was analyzed using ^{1}H-NMR. The synthesis is shown in **Figure 2.6** (Kurth et al 1987, Organikum, 2001, page 364).

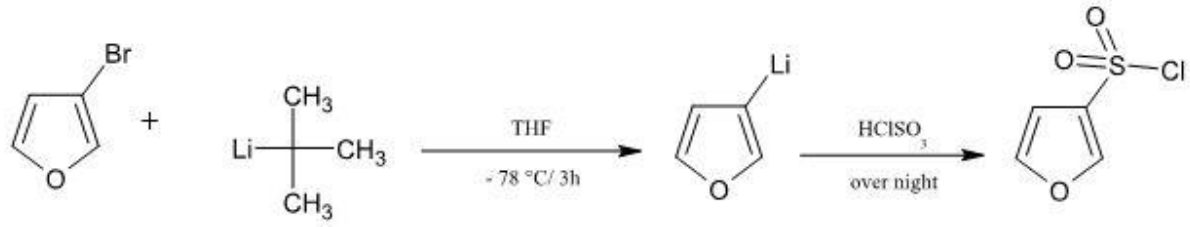


Figure 2.6 Synthesis of furan-3-sulfonyl chloride.

2.3.9 Synthesis of S-(2-furanyl)cysteine

The synthesis followed the similar approach used for the synthesis of S-(N-benzylpyrrol-2-yl)cysteine (described in section 2.3.1). Instead of N-benzylpyrrole, furan (0.04 ml, 50 mmol) was used in this reaction. Furan has a boiling point of about of 32°C and does not need to be heated up to 90°C. Unlike the synthesis of S-(N-benzylpyrrol-2-yl)cysteine, a colorless solution was obtained. After turning over the decanter, two phases (one whitish and water-soluble; one brown and methanol-soluble) were obtained (after Rudyakova et al., 2008). The mixture underwent ^{1}H-NMR and further analyses. The synthesis is shown in **Figure 2.7**.

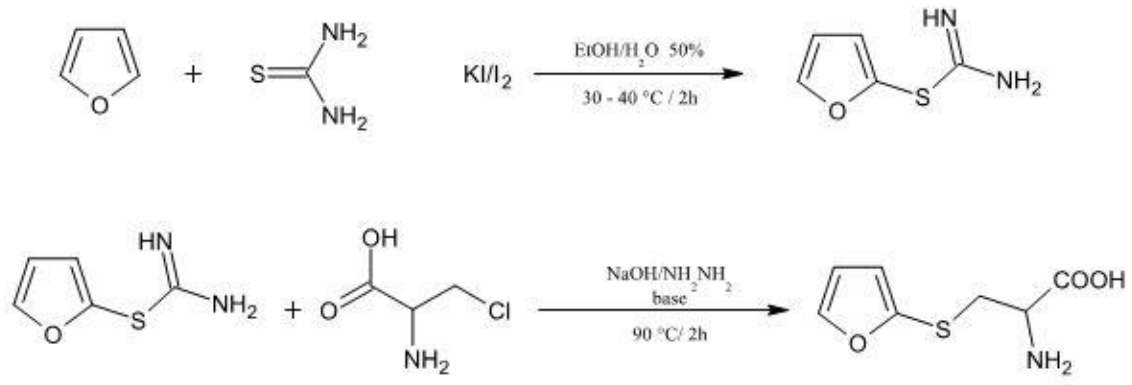


Figure 2.7 Synthesis reaction of S-(2-furanyl)cysteine.

2.3.10 Synthesis of N-Cbz-L-serine benzylester-O-trifilate

The purpose of the synthesis of this compound was to find an alternative compound to 3-chloro-L-alanine. N-Cbz-L-serine benzylester (329 mg, 1 mmol) in 0–2°C dry THF was added dropwise to 1.1 Eq trifluoromethanesulfonic anhydride 99% (0.18 ml, 1.1 mmol). The reaction was stirred for at least 30 min at RT. The volatile compounds were evaporated (at 40°C, 350 mbar). The solution was separated using a decanter in the mixture of ether and water. The organic phase was washed with water and HCl and consequently subjected to MS-EI analysis after evaporation. The synthesis is shown in **Figure 2.8** (Hirschmann et al., 1996).

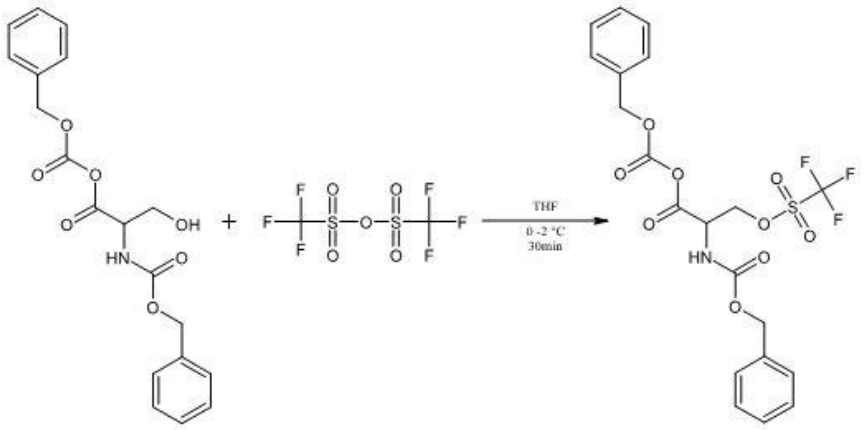


Figure 2.8 Synthesis of N-Cbz-L-serine benzylester-O-trifilate.

2.3.11 Synthesis of lithium furan-2-thiolate

Furan (14.40 ml, 200 mmol) was added to 70 ml dry THF under argon protection. t-Buli (58.8 ml, 100 mmol) in pentane was added portionwise to the reaction mixture. The temperature was increased to 35°C in the first 30 min and the reaction was stirred at RT for 2 h. Small portions of elemental sulfur (3.20 g, 100 mmol) were added and stirred at –20°C for 20 min; next, the solution was stored at –4°C for 24 h and evaporated (at RT, 250 mbar). The product obtained through the replacement of furan with lithium or sulfur (or sulfur-lithium) was analyzed using MS-EI. The synthesis is shown in **Figure 2.9** (after Holland et al., 1999).

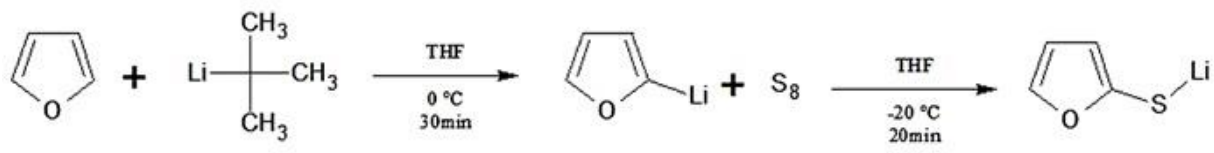


Figure 2.9 Synthesis of lithium furan-2-thiolate.

2.3.12 Synthesis of 2-[(tert-butoxycarbonyl)amino]-3-(1-*H*-pyrrole-2-ylsulfanyl)propanoic acid

To the suspension of Boc-cystine (1.20 g, 2.7 mmol) in 50 ml CH_2Cl_2 at –80°C, 0.13 ml bromide was added while stirring for 10–15 min. Pyrrole (0.38 ml, 5 mmol) and imidazole (340 mg, 5 mmol) dissolved in 10 ml CH_2Cl_2, were added to the first mixture and mixed for 30 min at 20–22°C. Purification of the reaction mixture was carried out using column chromatography (silica gel; 3 cm x 50 cm), which was eluted with 3% methanol in CH_2Cl_2. After the evaporation (at RT, 750 mbar) of CH_2Cl_2, the product was analyzed using TLC (after Zhang and Parkin, 2013). The synthesis is shown in **Figure 2.10**.

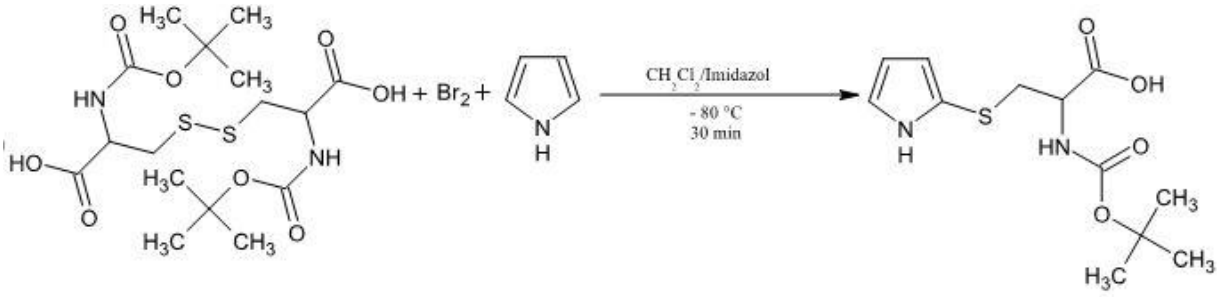


Figure 2.10 Synthesis of 2-[(tert-butoxycarbonyl)amino]-3-(1-H-pyttol-2-ylsulfanyl)propanoic acid.

2.3.13 Synthesis of 1-(triisopropylsilyl)pyrrole

Potassium (9.70 g, 248 mmol) in 500 ml THF was intensively heated up to 100°C. Pyrrole (17.20 ml, 248 mmol) was added dropwise (1 ml per min) to 100 ml THF. After 15 min at 100°C, the mixture was stirred at RT for 12 h. While stirring at 0–2°C, chlorotriisopropylsilane (47.80 g, 248 mmol) was added dropwise to the mixture. The reaction was stirred at RT for 12 h, filtered, and washed with THF several times. Next, THF was evaporated (at 30°C, 250 mbar) and the resultant colorless oil residue was analyzed using MS-ESI (after Stefan et al., 1989). The synthesis is shown in **Figure 2.11**.

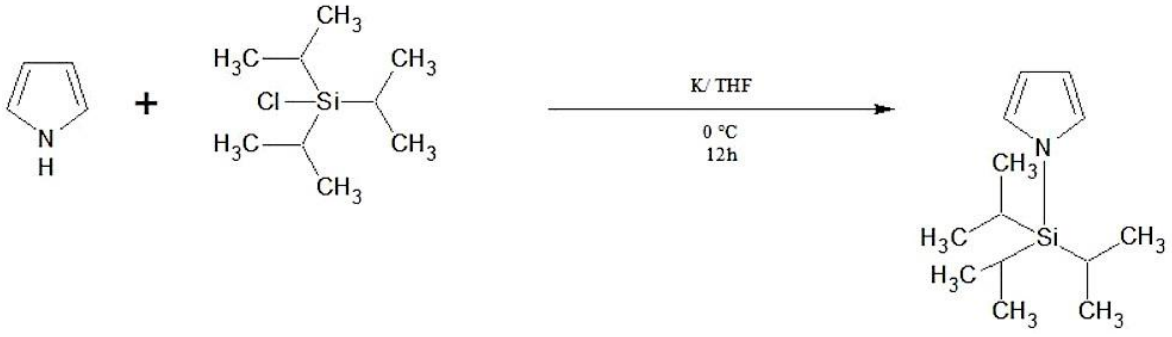


Figure 2.11 Synthesis reaction of 1-(triisopropylsilyl)pyrrole.

2.3.14 Synthesis of S-(2-pyrrolyl)cysteine using Allyl iso-thiocyanate

To a stirred solution of lithium diisopropylamide (1.50 g, 140 mmol) and t-BuOK (15:70 g, 140 mmol) in THF (60 ml), one solution of n-buLi (9.10 ml, 100 mmol) in 65 ml hexane was added under argon atmosphere at around –50˚C. A solution of allyl iso-thiocyanate (5.40 g, 50 mmol) in THF (40 ml) was added dropwise to the reaction during approximately 30 min at –20 to –15˚C. The reaction was stirred for 30 min at 45˚C; next, H_2O (8.00 g, 440 mmol) was added, intensively stirred for 20 min at approximately 30˚C. One portion of 3-chloro-L-alanine (14.00 g, 100 mmol) was further added and stirred for 40 min at 50˚C. The resultant reaction mixture was quenched on 100 ml ice, and aqueous phase was removed with pentane (2 × 30 ml) and Et_2O (2 × 30 ml).The organic phase was washed with H_2O (3 × 40 ml), dried with potassium carbonate (K_2CO_3), and evaporated (at RT, 750 mbar) (after Nedolya et al., 2013). The synthesis is shown in **Figure 2.12**.

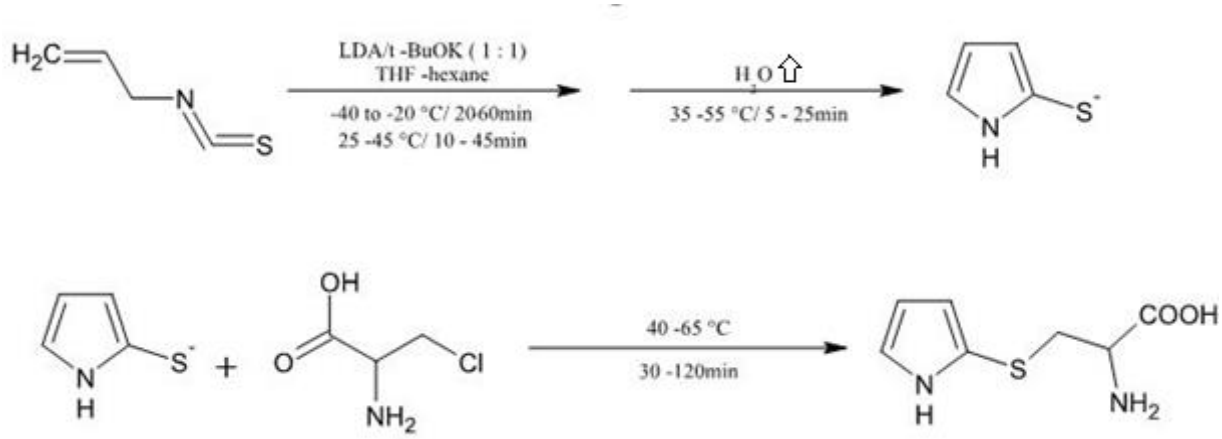


Figure 2.12 Synthesis of S-(2-pyrrolyl)cysteine.

2.3.15 Synthesis of S-(N-methylpyrrol-2-yl)cysteine

Method A) N-Methylpyrrole (0.05 ml, 0.5 mmol), N-Boc-cystine as disulfide (110 mg, 0.25 mmol) and copper(I) iodide or CuI (3 mol %) were mixed in DMSO (0.5 ml) and stirred for 20 h at 110°C. The reaction was evaporated (using high vacuum pump with liquid N2) and then analyzed.

Method B) N-methylpyrrole (0.05 ml, 0.5 mmol), N-Boc-cysteine (606 mg, 0.5 mmol) and copper(I) iodide or CuI (5 mol %), as the reaction catalyzer were mixed in DMSO (0.5 ml) and stirred for 20 h at 110℃. The reaction was evaporated (using high vacuum pump) and then analyzed (after Alves et al., 2012) using TLC. The synthesis is shown in **Figure 2.13**.

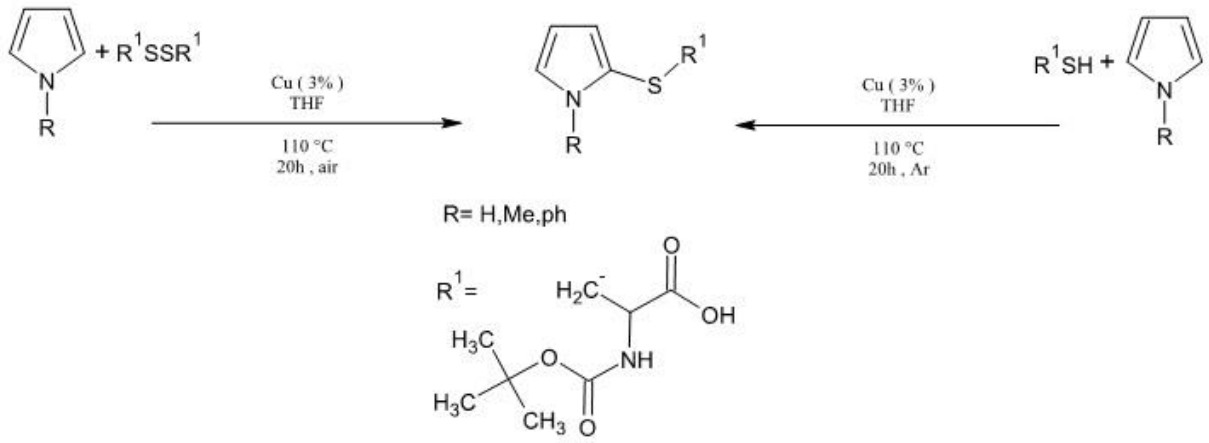


Figure 2.13 Synthesis of S-(N-methylpyrrol-2-yl)cysteine.

2.3.16 Synthesis of O-trifluoromethylsulfonyl-serine with triethylamine

Trifluoromethanesulfonic anhydride (3.00 g, 10.6 mmol) was added under argon atmosphere to 20 ml dichloromethane. L-Serine (1.11 g, 10.6 mmol) was added to the solution. When the solution was quenched to 0°C, a second solution of triethylamine (1.57 ml, 11 mmol) and dichloromethane (10 ml) was added dropwise. The reaction mixture was stirred at 0°C for 1 h. Dichloromethane was removed by evaporation (at 40°C, 1 atm). The residue was separated and purified by column chromatography (silica gel, 3 cm x 50 cm) (adopted from Ayad et al., 2005). TLC and MS-ESI were implemented to analyze the reaction mixture. The synthesis is shown in **Figure 2.14**.

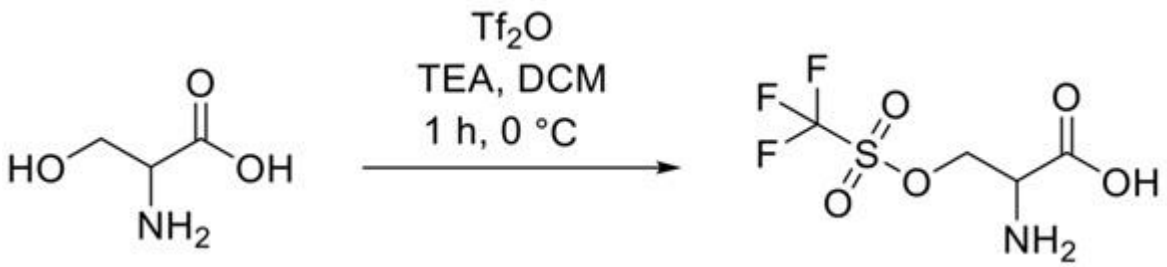


Figure 2.14 Synthesis of O-trifluoromethylsulfonyl-serine with triethylamine.

2.3.17 Synthesis of O-trifluoromethylsulfonyl-serine with 2,6-lutidine

A suspension of L-serine (66 mg, 0.63 mmol), 2,6-lutidine (134 mg, 1.25 mmol) and 50 ml dichloromethane was prepared under argon atmosphere. The suspension was stirred and the temperature was reduced to –40°C. Subsequently, a solution of trifluoromethanesulfonic anhydride (263 mg, 0.93 mmol) and 40 ml dichloromethane were added dropwise within 5 min. The mixture was stirred at –40°C for 1.5 h and analyzed using TLC (Kyasa et al., 2015). The synthesis is shown in **Figure 2.15**.

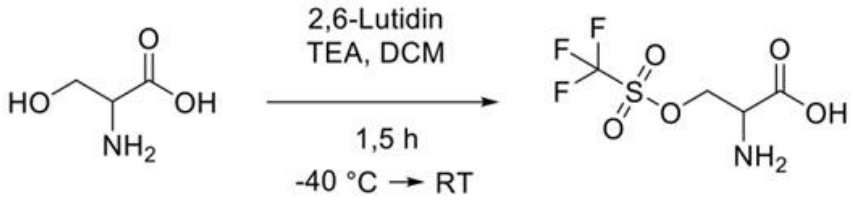


Figure 2.15 Synthesis of O-trifluoromethylsulfonyl-serine with 2,6-lutidine.

2.3.18 Synthesis of S-(3-thienyl)cysteine

In a three-necked flask, n-buli was added to the mixture of hexane (0.65 ml, 6.87 mmol), water-free THF (20 ml), and TMEDA (1.0 ml, 6.87 mmol). After cooling the solution mixture down to –78°C, 3-bromothiophene (0.56 ml, 6.24 mmol) was added dropwise in approximately 5 min. The reaction mixture was stirred at –78°C for 1 h. Subsequently, a suspension of L-cystin (1.65 g, 6.87 mmol) was produced, dried using THF (5 ml) and added dropwise to the reaction mixture over 10 min. The reaction was stored at RT for 4 h. Afterwards, the solution mixture was hydrolyzed with water (25 ml) and the organic phase was extracted using diethylether (3 x 20 ml). The extract was dried over Na_2SO_4 and the solvent was removed by a rotary evaporator under reduced pressure (40°C, 1 atm) (after Li et al., 2013, Nichols and Williard, 1993).

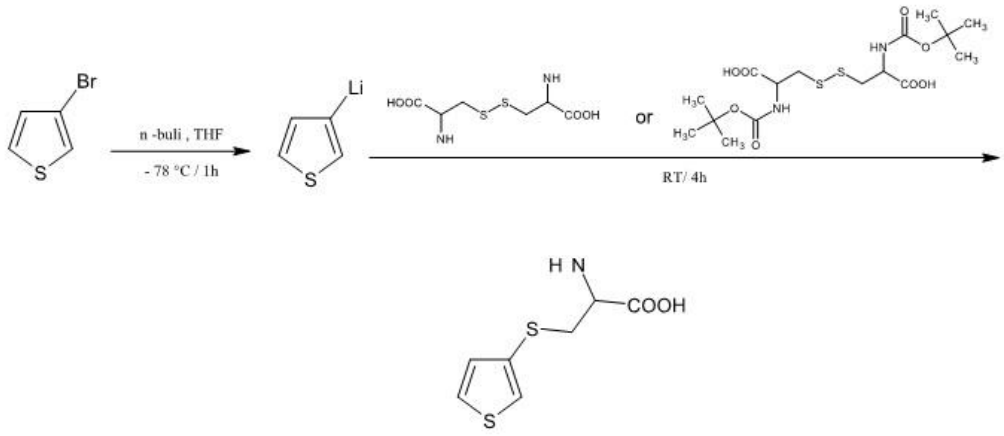


Figure 2.16 Synthesis of S-(3-thienyl)cysteine.

Boc-cystin was replaced by L-cystine, and the reaction followed as described above. The quantities of other reagents were chosen in accordance with the amount of Boc-cystine. The product was analyzed using ^{1}H-NMR and MS-ESI. The synthesis is shown in **Figure 2.16**.

2.3.19 Synthesis of 2-[(*tert*-butoxycarbonyl)amino]-3-[(1-methyl-1 *H*-pyrrol-2-yl)sulfanyl]propanoic acid

2-Bromo-N-methylpyrrole was synthesized first. N-Methylpyrrole (380 mg, 4.7 mmol) was dissolved in 20 ml THF and the solution was cooled down to –78°C. N-Bromosuccinimide (840 mg, 4.7 mmol) was added under argon protection. The reaction mixture was stirred at –78°C for 1 h and then mixed with triethylamine (470 mg, 4.7 mmol) and 50 ml cyclohexane. After being stirred for 5 min, the precipitate was filtered through a layer of Al_2O_3 (10.00 g, 98 mmol) and the solution dried with anhydrous Na_2SO_4. The solvent and the excessive amount of triethylamine were removed under reduced pressure (40°C, 90 mbar).

The next step started with the synthesis of N-(tert-Butoxycarbonyl)-S-(N-methylpyrrol-2-yl)cysteine. 2-Bromo-N-methylpyrrole (230 mg, 1.4 mmol) was obtained from the first step. The rest of the reaction was the same as the reaction described in section 2.3.18, which used 2-bromo-N-methylpyrrole instead of 3-bromothiophene (adopted from Dvornikova & Kamienska-Trela, 2002). The reaction mixture was analyzed using TLC and MS-ESI (+Q and –Q). The synthesis is shown in **Figure 2.17**.

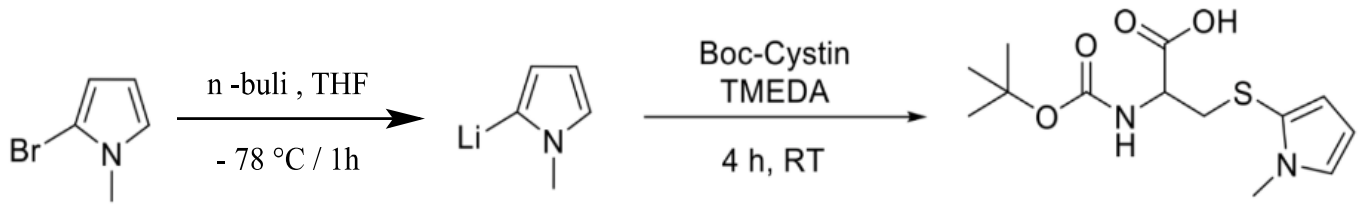


Figure 2.17 Synthesis of 2-[(tert-butoxycarbonyl)amino]-3-[(1-methyl-1 H-pyrrol-2-yl)sulfanyl]propanoic acid.

2.3.20 Synthesis of N- (*tert*-butoxycarbonyl)-O-trifluoromethylsulfonyl-serine with 2,6-lutidine

Under argon atmosphere, Boc-serine (307 mg, 1.5 mmol) was added to 10 ml dichloromethane. 2,6-Lutidine (0.19 ml, 1.65 mmol) and trifluoromethanesulfonic anhydride (0.27 ml, 1.65 mmol) were added dropwise at –40 °C over 5 min. After 20 min, the mixture was extracted with water and dichloromethane. The organic extract was dried over anhydrous magnesium sulfate and filtered. The solvent was removed under reduced pressure (40°C, 1 atm) (after Kyasa et al.,

2015). The solution was analyzed using TLC and ^{1}H-NMR. The synthesis is shown in **Figure 2.18**.

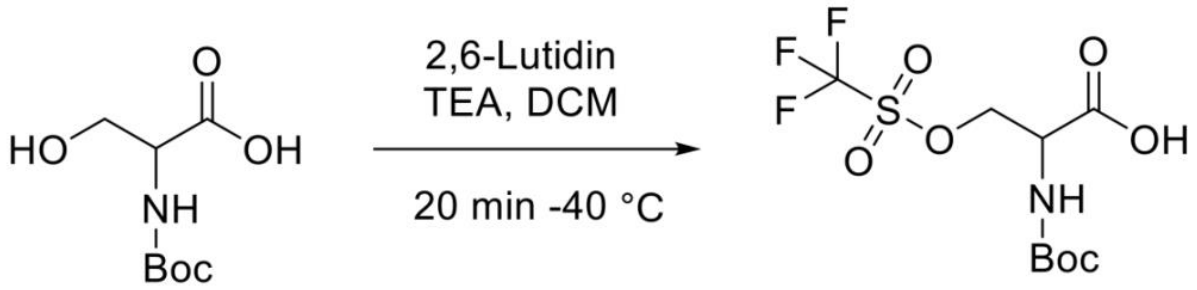


Figure 2.18 Synthesis of N-(tert-butoxycarbonyl)-O-trifluoromethylsulfonyl-serine with 2,6-lutidine.

2.3.21 Synthesis of S-(3-furanyl)cysteine

A 12.5 ml amount of dry ethanol in a three-necked flask was stirred with sodium under argon protection. After dissolving, sodium (242 mg, 105 mmol), NaOH (463 mg, 11.5 mmol), and L-cysteine (605 mg, 5 mmol) were added and stirred. The mixture was heated up to 100˚C for 10 min and 3-bromofurane (0.5 ml, 5.5 mmol) was added slowly, after which an orange precipitate formed. The reaction mixture was filtrated and the yellow residue was dissolved in 10 ml water and centrifuged for 10 min at 5,000 rpm at 3˚C. Following the acidification of the filtrate with acetic acid 70% (pH 5.5), it was mixed with 100 ml acetone and stored on ice overnight. After the yellow acetone solution evaporated (at RT, 480 mbar), a white solid mass appeared (after Keusgen, 1999). The reaction was analyzed using TLC and ^{1}H-NMR. The synthesis is shown in **Figure 2.19**.

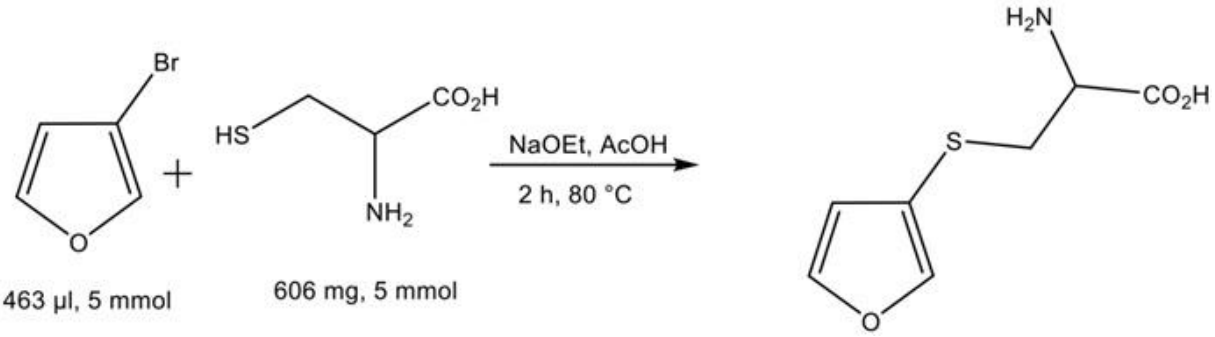


Figure 2.19 Synthesis of S-(3-furanyl)cysteine.

2.3.22 Synthetic of S-(N-triisopropylsilypyrrol-2-yl)cysteine

After thiourea (304 mg, 4 mmol) had fully dissolved in the mixture of water and ethanol (3 ml and 6 ml, respectively), triisopropylsylilpyrrol (446 mg, 2 mmol) was added slowly. A mixture of iodine (508 mg, 2 mmol) and potassium iodide (312 mg, 2 mmol) was added slowly to the water and ethanol (3 ml: 3 ml), until it turned green. The reaction mixture was stirred for 3 h at 35˚C. Next, NaOH (420 mg, 10.5 mmol) was dissolved in water and hydrazine hydrate 35 % (0.1 ml, 2 mmol) was dropped slowly into the mixture: as a result, the color transformed from greenish yellow to red. 3-Chloro-L-alanine (384 mg, 3.21 mmol) was dissolved in 5 ml water and added gradually to the reaction mixture, which was then heated up to 90–100˚C for 2 hr. The color remained unchanged after one hour. The mixture (with pH = 11) was washed, filtered over activated charcoal (1–2 g), acidified with HCL 10% to pH = 5.5 and stored overnight at 4˚C. A black precipitate was formed and the solvents were slowly removed using vacuum. The solution was centrifuged at 5000 rpm for 15 min and the black precipitate was cooled down to 4°C (after Rudyakova et al., 2008). TLC was performed to analyze the reaction. The synthesis is shown in **Figure 2.20**.

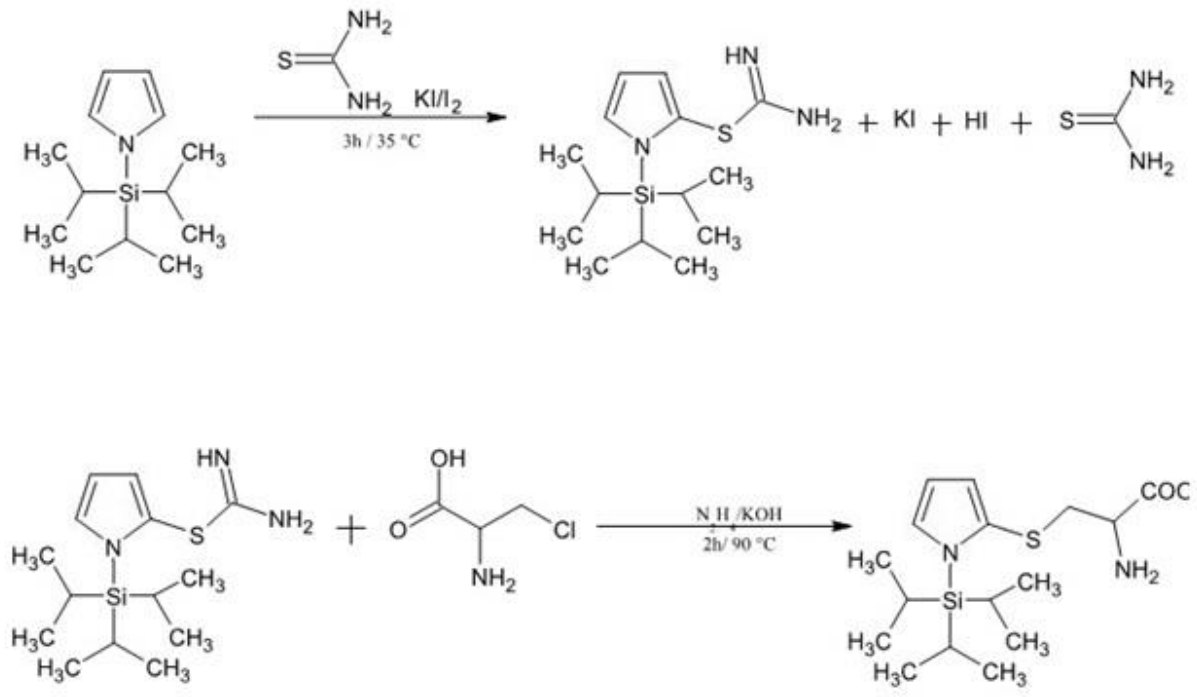


Figure 2.20 Synthesis of S-(N-triisopropylsilypyrrol-2-yl)cysteine.

2.3.23 Synthesis of S-(2-imidazolyl)cysteine

The reaction conditions were derived from the synthesis of the aliphatic S-alkenyl-l-cysteine (Keusgen, 1999). Argon was used as a protective gas. In a three-necked flask 20 ml DMF was dried using sodium (386 mg, 160 mmol). Potassium hydroxide (10 mmol) was dissolved under argon protection, and 2-imidazolethiol (200 mg, 2 mmol) were added to it. 3-Chloro-L-alanine (320 mg, 2 mmol) was mixed to the reagent and heated up to 100˚C for 2 hr. The solution was filtered and its pH adjusted to 5.5 with HCl 10%. Then it was evaporated (at 50˚C, 30 mbar) to reach 1 ml of total volume and precipitated using 10 ml acetone. To obtain crystals, the precipitate was washed with ethanol at 4˚C for 24 h (after Keusgen, 1999). Consequently, the reaction was analyzed using MS-ESI (+Q and –Q). The synthesis is shown in **Figure 2.21**.

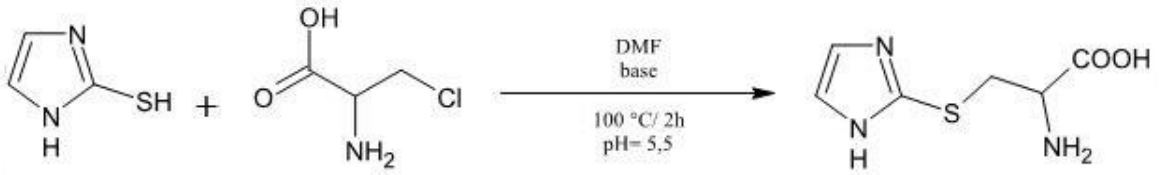


Figure 2.21 Synthesis of S-(2-imidazolyl)cysteine.

2.3.24 Synthesis of S-(N-methylimidazol-2-yl)cysteine

The reaction conditions were derived from the synthesis of the aliphatic S- alkenyl-l-cysteine (Keusgen, 1999). Argon was used as a protective gas. In a three-necked flask 20 ml, DMF was dried using sodium (386 mg, 0.16 mol). Potassium hydroxide (10 mmol) was dissolved in the solution under argon protection; next, 2-mercapto-1-methylimidazole (228 mg, 2 mmol) was added. 3-Chloro-L-alanine (320 mg, 2 mmol) was mixed to the reagent and heated up to 100˚C and then kept at that temperature for 2 hours. After the solution was filtered and its pH adjusted to 5.5 with HCl 10%, it was evaporated (at 50˚C, 30 mbar) to reach 1 ml of total volume. Precipitation was performed with 10 ml acetone. In order to obtain crystals, the precipitate was washed with ethanol and stored at 4˚C for 24 hr (after Keusgen, 1999). Subsequently, the reaction was analyzed using MS-ESI (+Q and –Q). The synthesis is illustrated in **Figure 2.22**.

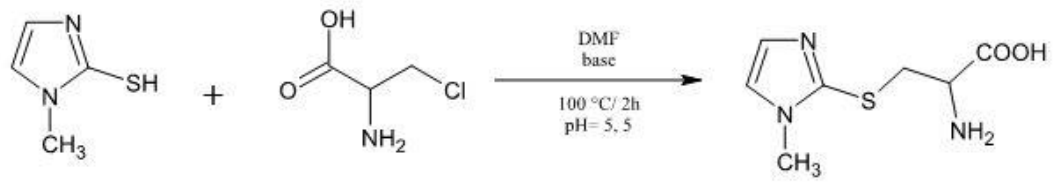


Figure 2.22 Synthesis of S-(N-methylimidazol-2-yl)cysteine.

2.3.25 Synthesis of S-(2-thiazolinyl)cysteine

The reaction conditions were derived from the synthesis of the aliphatic S- alkenyl-L-cysteine (Keusgen, 1999). Argon was used as a protecting gas. In a three-necked flask 20 ml, DMF was dried using sodium (386 mg, 0.16 mol). Potassium hydroxide (10 mmol) was dissolved under argon protection, after which 2-thiazolin-2-thiol (234 mg, 2 mmol) was added. 3-Chloro-L-alanine (320 mg, 2 mmol) was mixed with the reagent and refluxed for 2 hr. After the solution was filtered and its pH adjusted to 5.5 with HCl 10%, it was evaporated (at 50˚C, 30 mbar) to reach 1 ml of total volume and precipitated using 10 ml acetone. To obtain crystals, the precipitate was washed using ethanol at 4˚C for 24 h (after Keusgen, 1999). Subsequently, the reaction was analyzed using MS-ESI. The synthesis is illustrated in **Figure 2.23**.

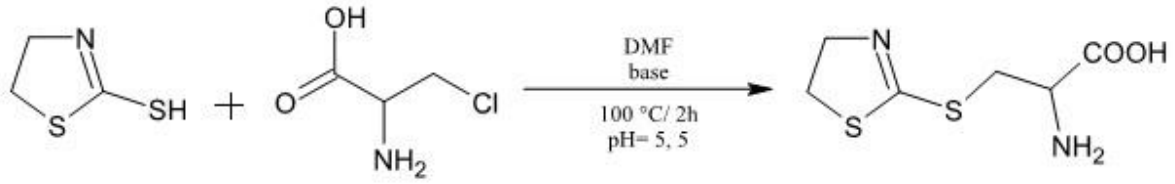


Figure 2.23 Synthesis of S-(2-thiazolyl)cysteine.

2.3.26 Synthesis of S-(3-thienyl)cysteine

With a slight modification, reaction was synthesized by the addition of potassium iodide and iodine (Rudyakova et al., 2008).

Thiourea (7.61 g, 100 mmol) was dissolved in 70 ml methanol, followed by the addition of KOH and 3-bromothiophene 97% (9.66 ml, 100 mmol). The solution was heated up to 100˚C for 3 h. Iodine (25.40 g, 100 mmol) and potassium iodide (16.60 g, 100 mmol) were dissolved in a mixture of ethanol and water (7 mL and 10 ml, respectively) and added to the reaction mixture. A white solid precipitate was dissolved after the mixture was heated to 70–80˚C. The solution was stirred for 7 h at 30–40˚C, while the mixture color remained unchanged. **Figure 2.24** illustrates the synthesis.

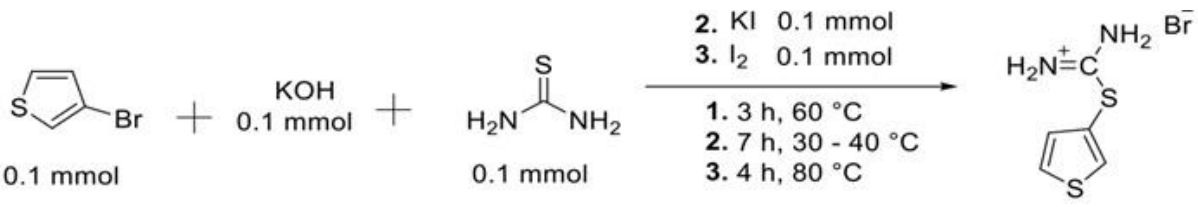


Figure 2.24 Synthesis of S-(3-thienyl)cysteine.

2.4 Oxidation

2.4.1 Oxidation of S-(N-benzylpyrrol-2-yl)cysteine

2.4.1.1 Synthesis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (oxidation with H_2O_2 in acetic acid)

1Eq reagent (2.58 g, 9.3 mmol) and 3/4 Eq H_2O_2 35% (0.6 ml, 7 mmol) were mixed with 3 ml acetic acid for 15 h under the light protection and the solvent was evaporated (at 40˚C, 30 mbar). Afterwards, it was dissolved in water and analyzed using MS-ESI.

2.4.1.2 Oxidation with $FeCl_3$ and H_5IO_6

Sulfide (358 mg, 1.3 mmol) solved in acetonitrile (3 ml, 0.04 mmol) was stirred for 5 min, after which 1.43 mmol H_5IO_6 was added. The excessive amount of H_5IO_6 was neutralized with $Na_2S_2O_3$ (3 g in 5 ml water). The mixture was washed with CH_2Cl_2 (4 x 5 ml) and the oil phase was dried with Na_2SO_4 by being gently turned over. The MS-ESI analysis using both the oil and the residue phases did not present acceptable results (after Kim et al. 2003).

2.4.1.3 Oxidation with Magnesium monoperoxyphthalate hexahydrate

Magnesium monoperoxyphthalate hexahydrate (MMPP) consists of water, ethanol, and methanol soluble. Sulfide (472 mg, 1.71 mmol) was added to ½ Eq MMPP (424 mg, 0.86 mmol). Methanol (3ml) was used as solvent. The solution was centrifuged and stored at 4°C. A white precipitate appeared in the solution after 2 h. TLC was performed separately to test the solution and the precipitate. The solvent has been evaporated (at 40˚C, 337 mbar) and dissolved in methanol for MS-ESI analysis (Mielke, 2015-doctoral thesis).

2.4.2 Oxidation of S-(N-methylpyrrol-2-yl)cysteine

2.4.2.1 Oxidation with H_2O_2 in water

The reaction was performed under argon atmosphere because of its high instability. 1 Eq S-(N-methylpyrrol-2-yl)cysteine (2.00 g, 10 mmol) was stirred in 2 ml H_2O and 1 Eq H_2O_2 35% (0.09 ml, 1 mmol) for 18 h. The reaction was controlled by TLC. The solvent was evaporated (at RT, 20 mbar) under argon protection in the next step and the water-soluble residue was analyzed using MS-ESI.

2.4.2.2 Oxidation with H_2O_2 in acetic acid

1 Eq S-(N-methylpyrrol-2-yl)cysteine (200 mg, 1 mmol) was fully dissolved in 3 ml acetic acid. 0.5 Eq H_2O_2 35% (0.6 ml, 0.7 mmol) was slowly added and stirred under argon protection for 15 h. In addition, TLC was performed to control the reaction's procedure. The solvent was evaporated (at 30˚C, 10 mbar) and the water-soluble residue underwent the MS-ESI analysis.

2.4.2.3 Oxidation with cumene hydroperoxide in H_2O

1 Eq S-(N-methylpyrrol-2-yl)cysteine (320 mg, 1.6 mmol) was dissolved in 3 ml water under argon protection. It was slowly stirred with 0.5 Eq cumene hydroperoxide (0.12 ml, 0.8 mmol) for 6 h. TLC confirmed the reaction progress. The solvent was evaporated (at RT, 20 mbar) and the water-soluble residue was subjected to MS-ESI analysis.

2.4.2.4 Oxidation with MMPP in H_2O

1 Eq S-(N-methylpyrrol-2-yl)cysteine (400 mg, 2 mmol) was fully dissolved in 4 ml water under argon protection. One-third of Eq MMPP (346 mg, 0.7 mmol) was added to the reagent and stirred thoroughly for 6 h. The reaction was analyzed by TLC. The solvent was evaporated (at RT, 20 mbar) afterwards and the water-soluble residue analyzed using MS-ESI (Mielke, 2015-doctoral thesis).

2.4.2.5 Oxidation with TAPC in H_2O_2 (35%)

1 Eq S-(N-methylpyrrol-2-yl)cysteine (200 mg, 1 mmol) was mixed with 0.1 Eq TAPC (35 mg, 0.1 mmol) as catalyzer. The reaction was performed by adding 0.1 ml H_2O_2 (35%). The solvent evaporated (at RT, 20 mbar) afterwards and the water-soluble residue underwent MS-ESI analysis (Bahrami et al., 2010).

2.4.3 Oxidation of S-(2-thienyl)cysteine with H_2O_2

1 Eq of S-(2-thienyl)cysteine (203 mg, 1 mmol) was dissolved in 3 ml H_2O and 1 Eq H_2O_2 35% (0.09 ml, 1 mmol**)** was added gradually to the reaction mixture. In-process control was performed using TLC analysis. The reaction was stirred for 12 h. The solvent was evaporated (at 40˚C, 60 mbar) until fully dried. ESI-MS, HNMR, IR, CNMR, HR-MS and TLC analyses were performed. The amount of product after oxidation was determined 105 mg = 0.48 mmol, which was fully dissolved in water.

2.5 Hydrogenation

2.5.1 Hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

2.5.1.1 Hydrogenation with pd/c and hydrogen

S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (137 mg, 0.47 mmol) was dissolved in 15 ml methanol with slight amount of catalytic 10% Pd/C and hydrogenated at a pressure of 4 bar for 3 h. The reaction mixture was centrifuged at 5,000 rpm for 10 min, after which the solution was evaporated (at RT, 280 mbar) and washed with acetone (20–30 ml) (Bhosale et al., 2012). The water-soluble product was analyzed using MS-ESI.

2.5.1.2 Hydrogenation with ammonium formate

Ammonium formate (1.53 g, 24.3 mmol) was added portionwise to S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (233 mg, 0.801 mmol) in combination with Pearlman's catalyst (129 mg) and ethanol (16 ml) under argon shield at RT. The reaction mixture was heated up to 100˚C for 2 h, cooled down to RT and passed through a pad of celite. The solution was filtrated, evaporated (at 40˚C, 175 mbar), and analyzed by MS (Fukuda et al., 2009).

2.5.2 Catalytic transfer hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (64 mg, 0.22 mmol) and ammonium formate (75 mg,1.2 mmol) were mixed with 4 ml ethanol and 0.5 ml water, and stirred under argon atmosphere at RT for 1 h after 10% Pd/C (40 mg) had been added. The reaction mixture was filtered over celite pad, evaporated (at 40˚C, 175 mbar), and analyzed by MS-ESI (Chadwick and Hodgson, 1983).

2.5.3 Hydrogenation of N-benzyl pyrrole

N-Benzylpyrrole 97% (0.318 ml, 2 mmol) was dissolved in 5 ml methanol, after which a slight amount (as big as spatula) of catalyst 10% Pd/C was added and hydrogenation performed at a pressure of 4 bar for 3 h. The reaction mixture was centrifuged (at 5,000 rpm, 4˚C), and the solvent was evaporated (at 40˚C, 175 mbar) and washed with acetone. The water-soluble reagent was conducted for ^{1}H-NMR analysis (Bhosale et al., 2012).

2.6 HPLC

2.6.1 Analytical HPLC method for identification and purification of synesthetic product S-(N-benzylpyrrol-2-yl)cysteine

The detailed description of the HPLC device is provided in **Appendix 2**. Water and methanol were used as an HPLC solvent with the column (EC 250/4. RT-NUCLEOSIL® 100-5 C18; flow rate: 0.7 mm/min). Optimal isocratic conditions to separate the desired product from the impurities and unwanted products were obtained by using the water-methanol mixture (60:40 *v/v*) (**Appendix 4**).

2.6.1.1 Preparative HPLC

Product purification and separation followed the same procedure as the analytical HPLC with the column (VP 250/10. RT-NUCLEODOR® C18 HTce, 5 µm; flow rate: 7 mm/min). The device is described in detail in **Appendix 2**.

2.6.2 Analytical HPLC method for identification and purification of synesthetic product S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

Water and methanol were used as an HPLC solvent with the column (EC 250/4. RT-NUCLEOSIL® 100-5 C18; flow rate: 0.7 mm/min). The optimal isocratic conditions were obtained by using the water-methanol mixture (63:37 *v/v*) (**Appendix 5**).

2.6.2.1 HPLC-MS

To ensure peak accuracy for a better purification, HPLC-MS analysis was carried out before the preparative HPLC under the condition described for analytical HPLC. The setting is described in detail in **Appendix 2**.

2.6.2.2 Preparative HPLC

This method was used to separate the oxidized and non-oxidized products from the unwanted products under similar chromatographic conditions as analytical HPLC with the column (VP 250/10. RT-NUCLEODOR® C18 HTce, 5 µm; flow rate: 7 mm/min).

2.6.3 Analytical HPLC method for identification of enzymatic activity test

Analytical HPLC (column: EC 250/4. RT-NUCLEODOR® C18 ce, 1 ml/min) has to be conducted to optimize the method for HPLC-MS analysis using water and methanol as an HPLC solvent (**Appendix 6**).

2.6.3.1 HPLC-MS

To determine the molecular weight of each visible peak in analytical HPLC, HPLC-MS analysis was performed (column: EC 250/2. NUCLEODOR® 100-5 C18 ce, 0.25 ml/min). Water and methanol mixture was used as a solvent and the device is described in detail in **Appendix 2**.

2.7 Alliinase enzymatic activity test

The enzymatic activity of alliinase was tested on six synthesized products to determine whether they show substrate specificity to alliinase. The products obtained from alliinase reaction with each of the synthesized products were analyzed using HPLC-MS. Two out of six products were substrate specific for aliinase and four remained non-oxidized.

2.7.1 Alliinase enzymatic activity test on S-(2-thienyl)cysteine-S-oxide

HPLC-MS was conducted to estimate the optimal reaction time and conditions for the analysis, which resulted in separation and identification of the obtained compounds. The most stable compound S-(2-thienyl)cysteine-S-oxide was selected for the enzymatic digestion. In 2 ml Eppendorf Snap-Cap, 50 mg of S-(2-thienyl)cysteine-S-oxide was dissolved in 0.5 ml H_2O. The pH was adjusted to 7–7.4 for *A. sativum* alliinase by adding 0.5 ml of alliinase buffer (**Appendix 3**). A 50 µl amount of alliinase (Krest & Keusgen, 1999b) was then added to the Eppendorf cap. As a positive control, 50 mg alliin was dissolved in 0.5 ml H_2O in the second tube. The pH was set to the optimum by adding 0.5 ml of buffer and 50 µl of enzyme. In the third tube, as a negative control, 50 µl enzyme was added to 1 ml H_2O. As organic phase, 1 ml cyclohexane was added to each tube. The organic layer was analyzed after 2, 4, and 6 hours. Later, 1 ml of cyclohexan phase was separated and dried using 50 mg $MgSO_4$. It was evaporated (at RT, 180 mbar), reducing volume to approximately 200 µl. The mixture was analyzed with HPLC-MS after the optimization method using analytical HPLC (**Appendix 2**). A column (RT-NUCLEOSIL® 100-5 C18) was used for HPLC. An optimal approach is represented in **Appendix 6**. Details of HPLC-MS analysis are shown in **Appendix 2**.

2.7.2 Alliinase enzymatic activity test on the other five main products

The analysis was also performed on the other five products as mentioned above. In each tube, 50 mg of each compound was dissolved in 0.5 ml H_2O and pH was optimized (7–7.4) by adding 0.5 ml alliinase buffer. A 50 µl buffer of alliinas extracted from *A. sativum* was added to each tube in addition to 1 ml organic phase. The reaction mixture was incubated for 2 h while being shaken. After 2 h, organic phase was separated and dried using 50 mg $MgSO_4$. Afterwards, the solvent was evaporated as mentioned in section 2.7. annd HPLC-MS analysis was conducted.

3. Results

In this research, 3-chloro-L-alanine hydrochloride was used as the primary compound for the synthesis of other products that are combined with the amino acid cysteine and 5-ring aromatic heterocycles including pyrrole-compounds and thiophene rings. The results of the analyses of these products, (3-chloro-L-alanine, S-(N-benzylpyrrol-2-yl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, S-(2-pyrrolyl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine S-oxide, S-(2-thienyl)cysteine and S-(2-thienyl)cysteine-S-oxide), which were carried out using TLC, C-NMR, H-NMR, MS, and HR-MS are listed in **Table 3.1(a,b)** and **Figs. 3.1–3.35**. All NMR spectra details including signals such as δ, chemical shift, ppm and J, coupling constant, Hz, as well as peaks and splits are illustrated in **Table 3.1(a)**. The obtained multiplicities include: singlet; d: doublet; dd: doublet of doublets; and md: multiple of doublets. The numbers highlighted in the molecular structure next to NMR spectra correspond to the number of hydrogen and carbon atoms listed in **Table 3.1(a)** and **3.1(b**). The results of IR spectra and ESI-MS analyses including stretching bands and molecular weight of each product are shown in **Table 3.2** The results of the methods that fully or partially failed are presented in the **Appendixes (18 – 52)** of the method section. **Appendixes 4–5** contain analytical and preparative HPLC results.

3.1 Synthesis of 3-chloro-L-alanine hydrochloride

3-Chloro-L-alanine hydrochloride, having an estimated yield of 86%, was synthesized first as the main primary compound used in synthesis of other products.

3.1.1 TLC analysis

3-Chloro-L-alanine has no chromophore; thus, it cannot be detected under UV light. The results indicate that serine was used as the main, primary educt, fully consumed in the reaction and fully transformed to alanine. The Rf at 0.125 was calculated for L-serein. In the product line, the main purple spot appeared with the Rf at 0.38 (**Figure 3.1**)

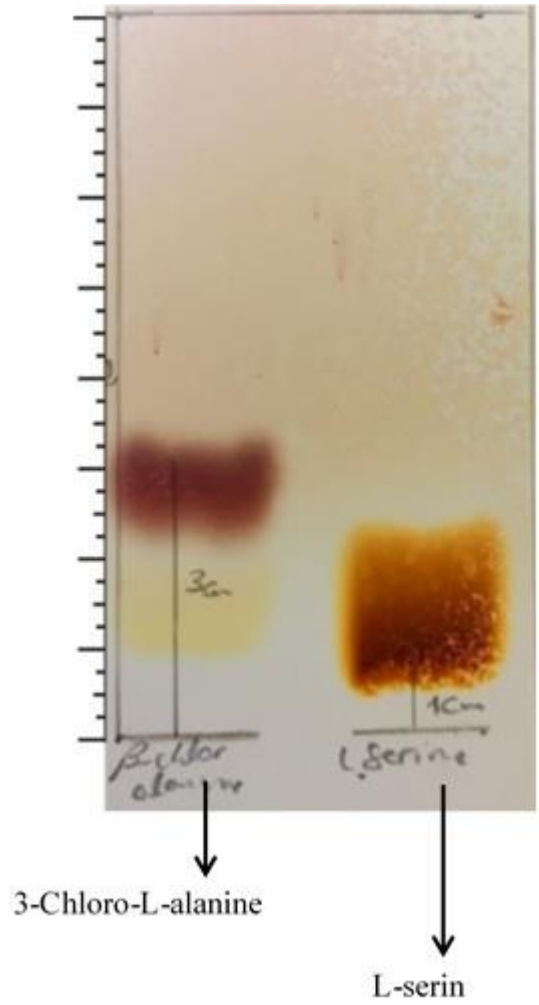
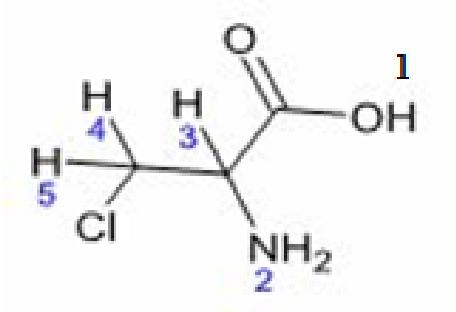
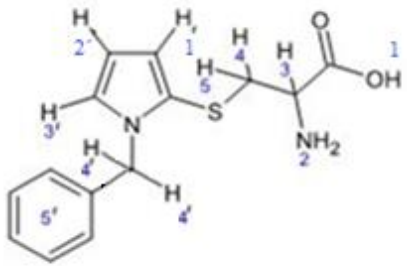


Figure 3.1 Silica thin-layer chromatography (TLC) of 3-chloro-L-alanine using ninhydrin reagent for detection.

3.1.2 ^{1}H-NMR analysis

As shown in **Table 3.1(a)** and **Figure 3.2**, the spectrum illustrates 3 signals all with integral 1, which indicates the existence of 3-chloro-L-alanine. The methin hydrogen group at the chiral center has a signal at δ = 4.45 ppm (*J* = 3.43, 4.35 Hz, dd). Two distinguishable diastereotopic methylene hydrogens in the spectrum include H4 δ = 4.00 ppm (*J* = 3.43, 12.82 Hz, dd) and H5 δ = 4.11 ppm (*J* = 4.35, 12.82 Hz, dd).

Table 3.1(a) ^{1}H NMR analyses of six main synthesized products.

		^{1}H NMR			
Products	**Hydrogen nr.**	**Chemical shift (in ppm)**	**Multipilicity**	**Coupling constent J [Hz]**	**Number of hydrogen atoms**
3-Chloro-L-alanine					
	1				
	2				
	3	4.45	dd	4.35, 3.43	1
	4	4.00	dd	12.80, 3.43	1
	5	4.11	dd	12.80, 4.35	1
S-(N-benzylpyrrol-2-yl)cysteine					
	1				
	2				
	3	3.498	dd	3.21, 10,53	1
	4	2.63	dd	10,53, 14,43	1
	5	3.06	dd	3.21, 14,43	1
	1´	6.144	dd	2.98, 3.66	1
	2´	6.524	dd	1.83, 3.66	1
	3´	6.949	dd	1.83, 2.98	1
	4´	5.315	dd	15.57, 39.15	2
	5´	7.25	md		5

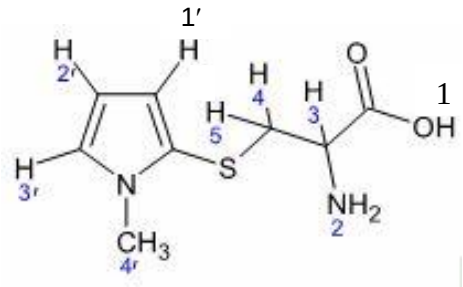
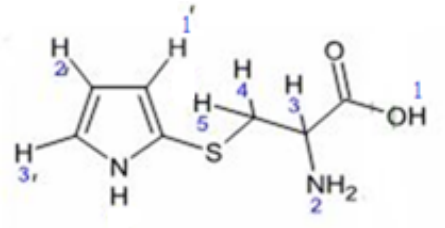
		^{1}H NMR			
Products	**Hydrogen nr.**	**Chemical shift (in ppm)**	**Multipilicity**	**Coupling constent J [Hz]**	**Number of hydrogen atoms**
S-(N-methylpyrrol-2-yl)cysteine					
	1				
	2				
	3	3.785	dd	8.70, 3.43	1
	4	3.00	dd	8.70, 14,43	1
	5	3.66	dd	5.40, 14.43	1
	1´	6.20	dd	2.75, 5.72	1
	2´	6.51	dd	1.14, 2.98	1
	3´	7.02	md		1
	4´	3.76	s		3
S-(2-pyrrolyl)cysteine					
	1				
	2				
	3	3.85	dd	3.89, 8.70	1
	4	3.11	dd	8.70, 14.65	1
	5	3.36	dd	3.66, 14.65	1
	1´	6.319	dd	3.21, 1.60	1
	2´	6.550	dd	1.60, 3.43	1
	3´	7.095	dd	1.60, 2.75	1

Table 3.1(a) continued

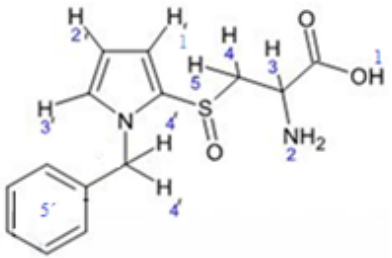
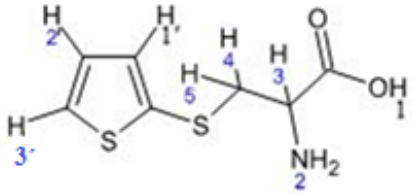
		^{1}H NMR			
Products	**Hydrogen nr.**	**Chemical shift (in ppm)**	**Multipilicity**	**Coupling constent J [Hz]**	**Number of hydrogen atoms**
S-(N-benzylpyrrol-2-yl)cysteine S-oxide					
	1				
	2				
	3	3.95	dd	4.58, 7.33	1
	4	3.36	dd	8.01, 13.97	1
	5	3.67	dd	4.58, 13.97	1
	1´	7.146	md		1
	2´	6.42	md		1
	3´	7.04	d	7.33	1
	4´	5.373	md		2
	5´	7.3	md		5
S-(2-thienyl)cysteine					
	1				
	2				
	3	3.75	dd	8.70, 3.89	1
	4	3.15	dd	14.65, 8.70	1
	5	3.40	dd	14.65, 3.89	1
	1´	7.07	dd	5.27, 3.66	1
	2´	7.30	dd	3.66, 1.37	1
	3´	7.56	dd	5.27, 1.37	1

Table 3.1(a) continued

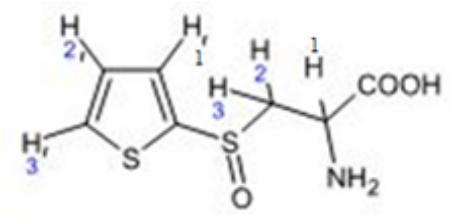
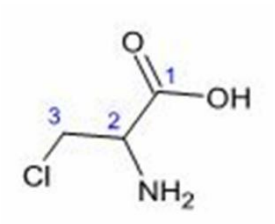
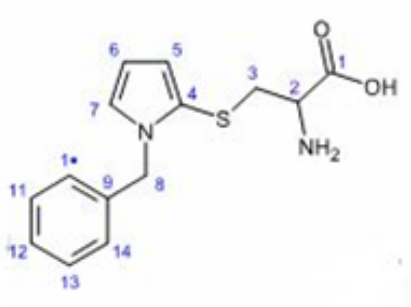
		^{1}H NMR			
Products	**Hydrogen nr.**	**Chemical shift (in ppm)**	**Multipilicity**	**Coupling constent J [Hz]**	**Number of hydrogen atoms**
S-(2-thienyl)cysteine-S-oxide					
	1	4.16	md	2.52,9.62	1
	2	3.65	md	2.52,6.41	1
	3	3.91	md	5.72,9.62	1
	1	7.41	md		1
	2	7.23	md		1
	3	7.93	md		1

Table 3.1(b) ^{13}C NMR analyses of six main synthesized products.

		^{13}C NMR
Products	**Carbon nr.**	**Chemical shift (in ppm)**
3-Chloro-L-alanine		
	1	169.04
	2	54.03
	3	42.11
S-(N-benzylpyrrol-2-yl)cysteine		
	1	171.518
	2	53.528
	3	37.898
	4	117.645
	5	119.101
	6	108.340
	7	125.656
	8	49.972
	9	126.60
	10	128.08
	11	126.94
	12	128.358
	13	127.112
	14	138.861

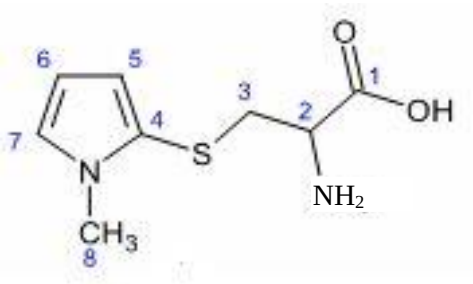
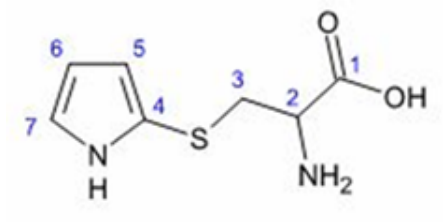
		^{13}C NMR
Products	**Carbon nr.**	**Chemical shift (in ppm)**
S-(N-methylpyrrol-2-yl)cysteine		
	1	172.56
	2	53.748
	3	36.92
	4	117.913
	5	118.431
	6	108.32
	7	127.29
	8	33.826
S-(2-pyrrolyl)cysteine		
	1	172.57
	2	54.150
	3	34.851
	4	123.00
	5	117.376
	6	109.624
	7	119.657

Table 3.1(b) continued

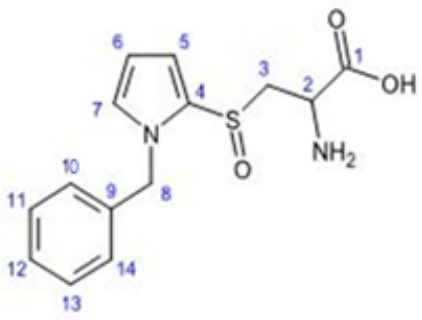
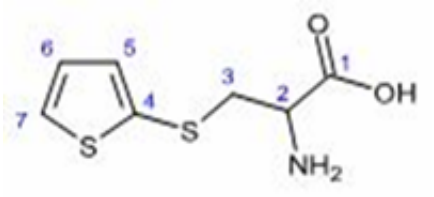
	^{13}C NMR	
Products	**Carbon nr.**	**Chemical shift (in ppm)**
S-(N-benzylpyrrol-2-yl)cysteine S-oxide		
	1	171.94
	2	47,28
	3	56.36
	4	137.49
	5	115.22
	6	105.88
	7	120.69
	8	49.32
	9	127.00
	10	128.80
	11	127.50
	12	129.30
	13	127.10
	14	135.00
S-(2-thienyl)cysteine		
	1	173.12
	2	53.84
	3	30.58
	4	135.60
	5	128.42
	6	131.42
	7	130.90

Table 3.1(b) continued

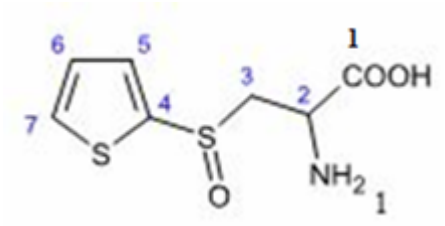
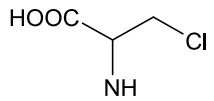
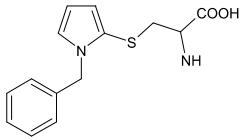
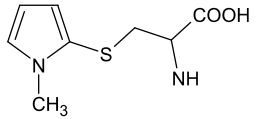
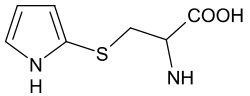
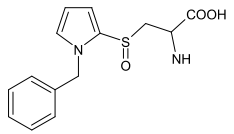
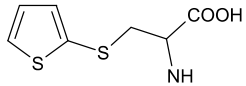
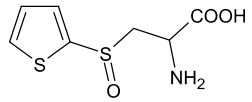
		^{13}C NMR
Products	**Carbon nr.**	**Chemical shift (in ppm)**
S-(2-thienyl)cysteine-S-oxide		
	1	171.04
	2	54.72
	3	56.88
	4	140.90
	5	129.88
	6	127.40
	7	132.90

Table 3.2 IR and ESI-MS analyses of the synthesized products.

	IR						ESI-MS				
	C-H Alkane (Cm-1)	**C-H Aromat (Cm-1)**	**C=C Alromat (Cm-1)**	**C=O (Cm-1)**	**OH (Cm-1)**	**NH2 (Cm-1)**	**$[M+H]^+$**	**$[M+Na]^+$**	**$[M+K]^+$**	**$[2M+H]^+$**	**Molecular Structure**
3-Chloro-L-alanine	2704	—	—	1739	3200	2873 & 2950	124.03	—	—	247.09	
S-(N-benzylpyrrol-2-yl)cysteine	2900	3025	1427	1605	3606	3200 & 3300	277.09	299.10	—	553.30	
S-(N-methylpyrrol-2-yl)cysteine	2950	3000	1457	1614	3242	3214 & 3156	201.06	223.08	239.07	—	
S-(2-pyrrolyl)cysteine	2900	3000	1388	1608	3300	3134	187.13	209.01	—	—	
S-(N-benzylpyrrol-2-yl)cysteine S-oxide	2603	2900	1496	1706	3400	2927 & 3100	293.15	315.15	331.17	—	

	IR						ESI-MS				
	C-H Alkane (Cm-1)	**C-H Aromat (Cm-1)**	**C=C Alromat (Cm-1)**	**C=O (Cm-1)**	**OH (Cm-1)**	**NH2 (Cm-1)**	**$[M+H]^+$**	**$[M+Na]^+$**	**$[M+K]^+$**	**$[2M+H]^+$**	**Molecular Structure**
S-(2-thienyl)cysteine	2600	2950	1510	1581	3450	2977 & 3100	204.08	—	242.30	—	
S-(2-thienyl)cysteine-S-oxide	2900	2992	1496	1626	3380	3088 & 3250	220.07	242.10	258.08	—	

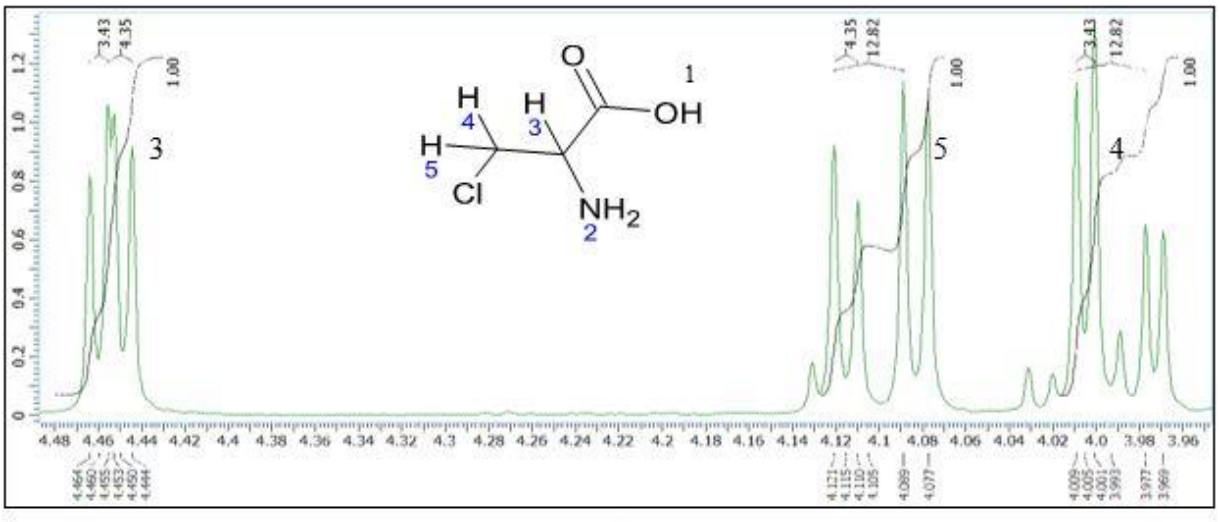


Figure 3.2 [1]H NMR spectra of 3-chloro-L-alanine.

3.1.3 [13]C-NMR analysis

This compound formed of three atoms of carbon and hereby three signals were observed.

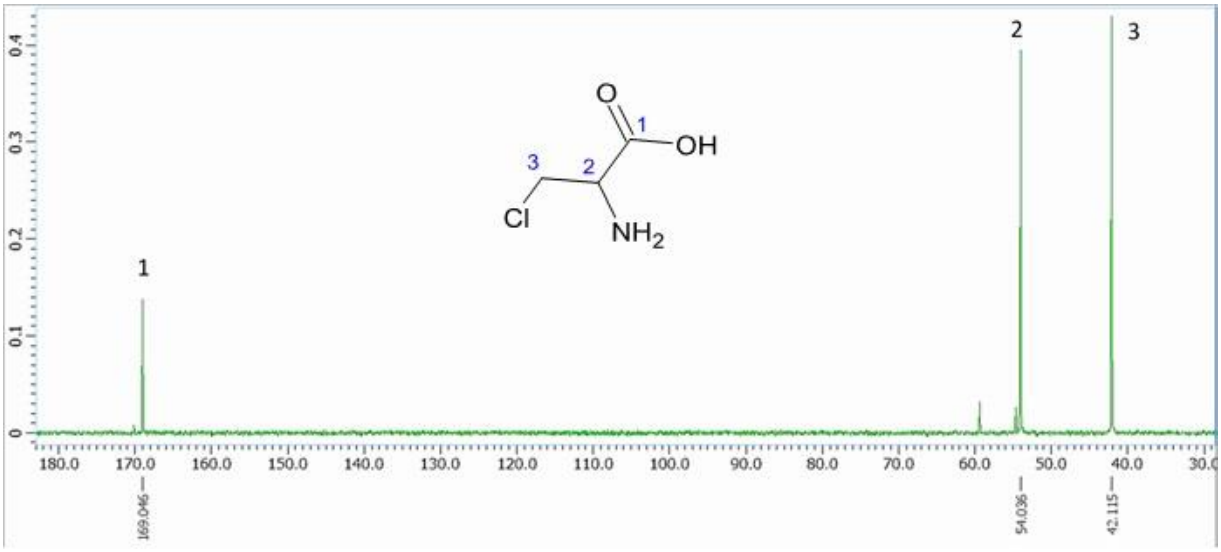


Figure 3.3 [13]C NMR spectra of 3-chloro-L-alanine.

The signal attributed to carboxyl carbon was recognizable at δ=169.04 ppm. In addition, carbons 2 at δ=54.036 ppm and 3 δ=42.115 ppm was readily visible (**Table 3.1(b)** and **Figure 3.3)**.

3.1.4 IR analysis

The IR analysis revealed several stretching bands in the IR spectrum at 1739, 2704, 2873, 2950 and 3200 cm^{-1}. The strong band at 1739 cm^{-1} corresponded to the carbonyl C=O group, and the weak band at 2704 cm^{-1} was related to C-H in the alkane group. The other weak bands corresponding to the NH_2 group were visible at 2873 and 2950 cm^{-1}. In addition, the presence of the hydroxyl (OH) group displayed a weak band at 3200 cm^{-1} (**Figure 3.4**).

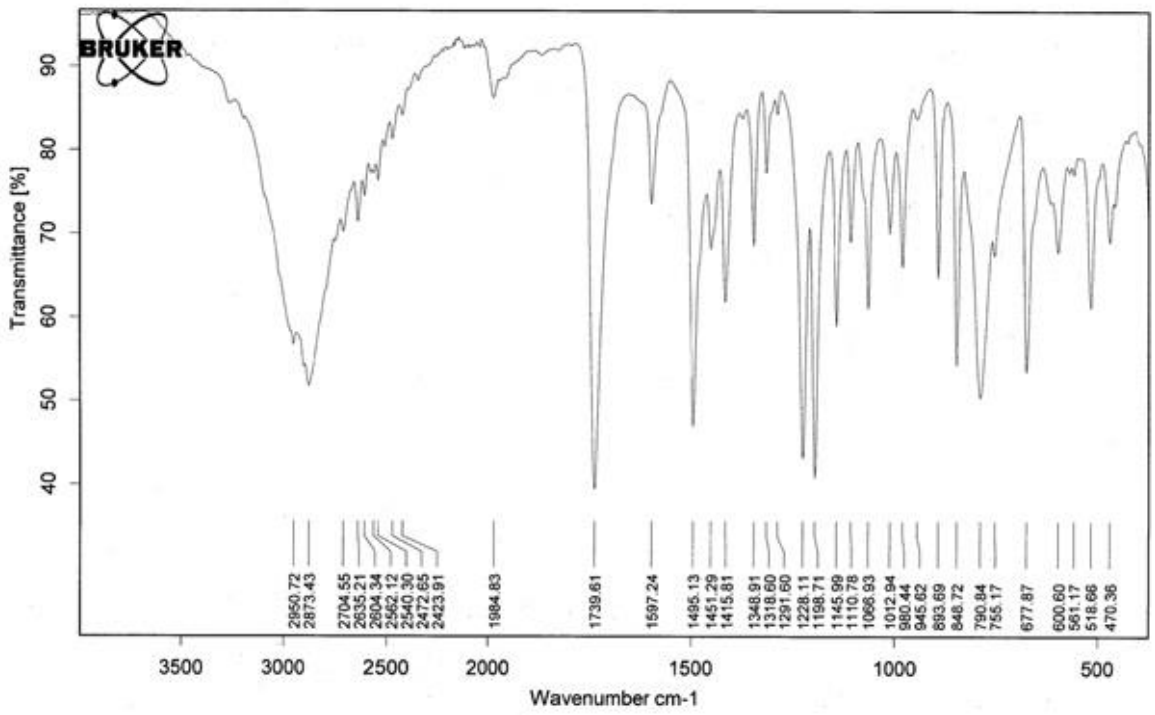


Figure 3.4 IR spectrum of 3-chloro-L-alanine.

3.1.5 ESI-MS analysis

Considering the ESI-MS analysis, the molecular weights assigned to 3-chloro-L-alanine were *m/z* 124 $[M+H]^+$ and *m/z* 126 $[M+H]^+$, in which the chloride was represented by the isotopes Cl = 35.5 g/mol and Cl = 37.5 g/mol, respectively (**Figure 3.5**).

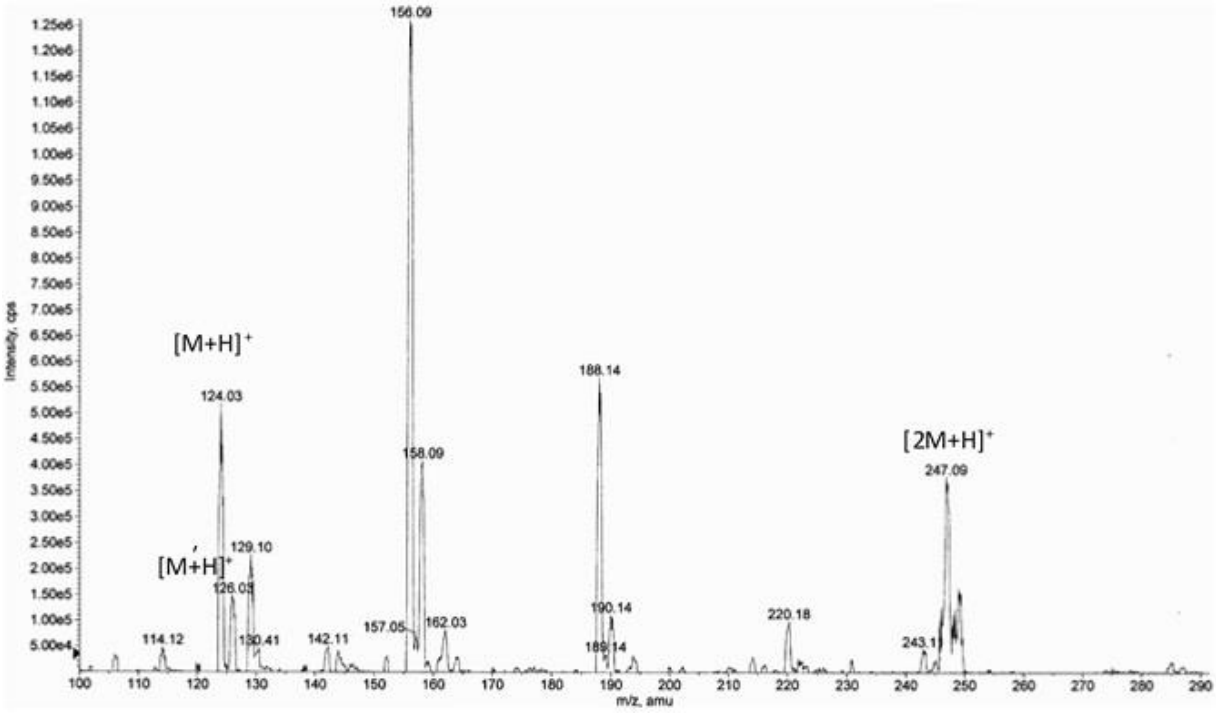


Figure 3.5 ESI-MS analysis of 3-chloro-L-alanine.

3.1.6 HR-MS analysis

Regarding HR-MS, the analysis of the accurate mass shows a molecular weight of *m/z* 124.0149 $[M+H]^+$ corresponding to the formula $C_3H_7ClNO_2$, consistent with the proposed structure (*m/z* 124.0160) that differs by 1.1 mDa (**Figure 3.6**).

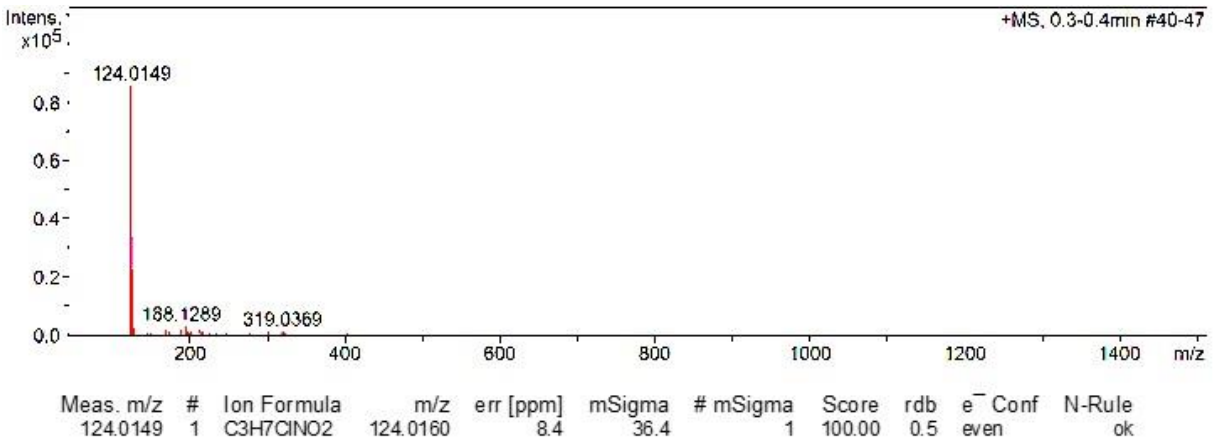


Figure 3.6 HR-MS analysis of 3-chloro-L-alanine.

3.2 Synthesis of S-(N-benzylpyrrol-2-yl)cysteine

As the N-benzylpyrrole group is more stable than the free pyrrole, the compound S-(N-benzylpyrrol-2-yl)cysteine can be used prior to the synthesis of S-(2-pyrrolyl)cysteine S-oxide, the yield of which is 47%.

3.2.1 TLC analysis

Using 3-chloro-L-alanine and thiourea as references, TLC was successfully performed in two steps. Product and thiourea spots were observed under the UV light at Rf = 0.63 and Rf = 0.64, respectively. Moreover, the spot of 3-chloro-L-alanine was formed at Rf = 0.38 after the plate had been sprayed by ninehydrin. In the product line, no 3-chloro-L-alanine was detected and the product was fully reacted (**Figure 3.7**).

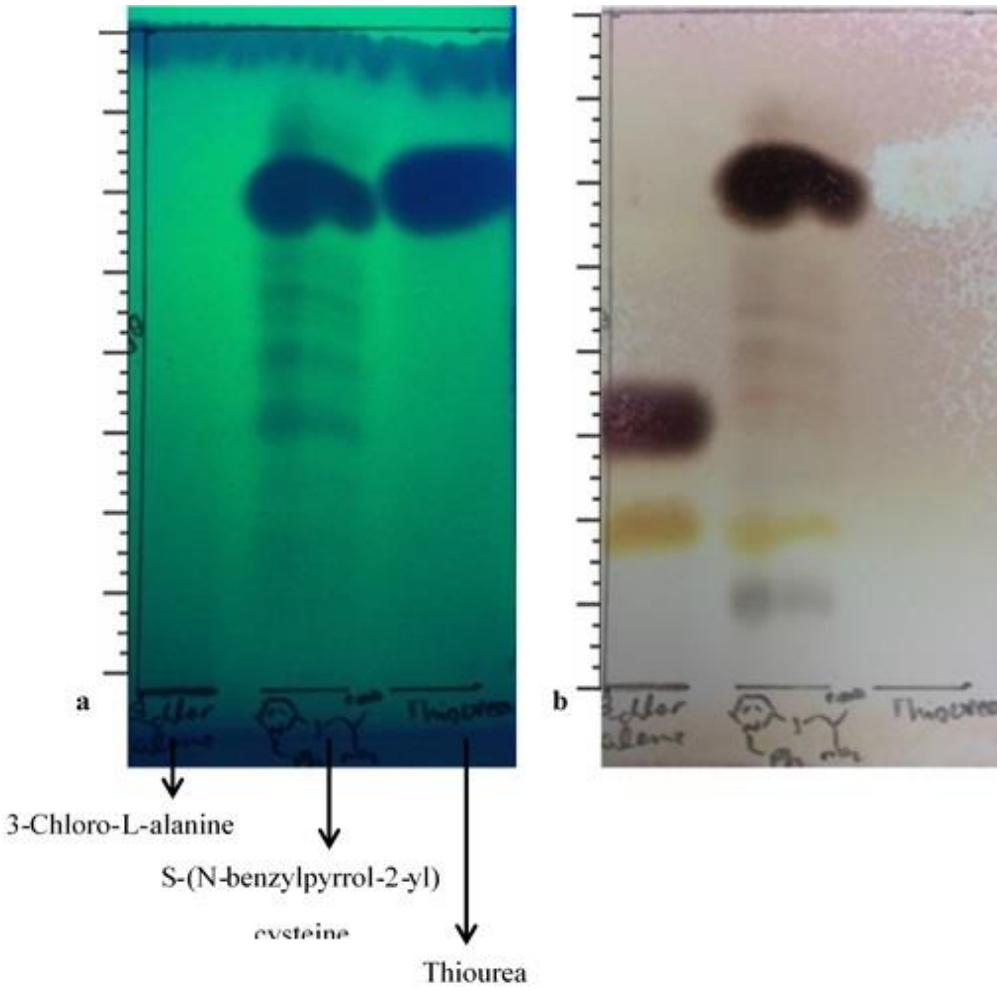


Figure 3.7 Analysis by TLC of S-(N-benzylpyrrol-2-yl)cysteine: a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

3.2.2 ^{1}H-NMR analysis

From **Figure 3.8**, which contains the ^{1}H-NMR spectra obtained in CD_3OD, one can observe the following details: a) in the aliphatic part of the molecule, H3 with integral 1 at δ = 3.498 ppm (J = 3.21, 10.53 Hz, dd); b) diastereotopic methylenee hydrogens 4 and 5, each with integral 1 at δ = 2.63 ppm (J = 10.53, 14.43 Hz, dd) and δ = 3.06 ppm (J = 3.21, 14.43 Hz, dd), respectively; c) methylene hydrogens H_4' of the benzyl group δ = 5.315 ppm (*J* = 15.57, 39.15 Hz, dd) with integral 2; and d) hydrogens 1′, 2′ and 3′ of the pyrrol group each with integral 1 at δ = 6.14 ppm (*J* = 2.98, 3.66 Hz, dd), δ = 6.52 ppm, (*J* = 1.83, 3.66 Hz, dd), and δ = 6.945 ppm (*J* = 1.83, 2.98 Hz, dd), respectively. All hydrogens belonging to the benzyl group were multiplets and were observed at δ = 7.2 ppm with integral 5 (**Table 3.1(a)** and **Figure 3.8**).

3.2.3 ^{13}C-NMR analysis

This analysis was conducted using CD_3OD as a solvent and fourteen strong signals were observed in the ^{13}C-NMR spectrum. The carboxylic carbon showed a characteristic signal at δ = 171.5 ppm. Also, methin carbon and the methylene carbon in the aliphatic part were identified at δ = 53.5 ppm and at δ = 37.89 ppm, respectively. The signal at δ = 49.97 ppm was related to benzyl carbons. Carbons 4, 5, and 6, which correspond to the pyrrole group showed chemical shifts at δ = 117.64 ppm, δ = 119.01 ppm and δ = 108.340 ppm, respectively. The carbons of the benzyl group indicated a typical chemical shift at δ = 125–138 pm. (**Table 3.1(b)** and **Figure 3.9**).

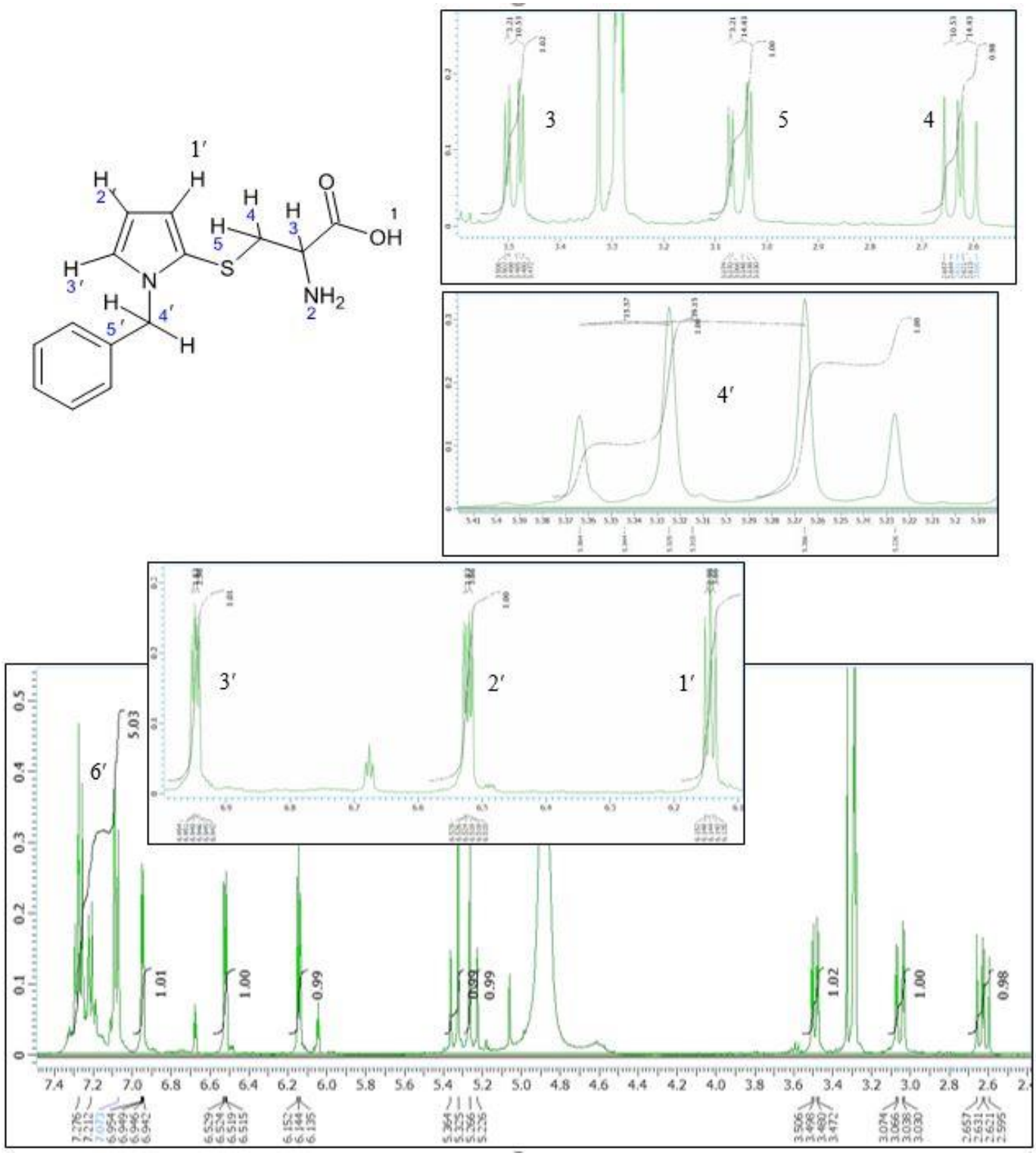


Figure 3.8 ^{1}H NMR spectra of S-(N-benzylpyrrol-2-yl)cysteine

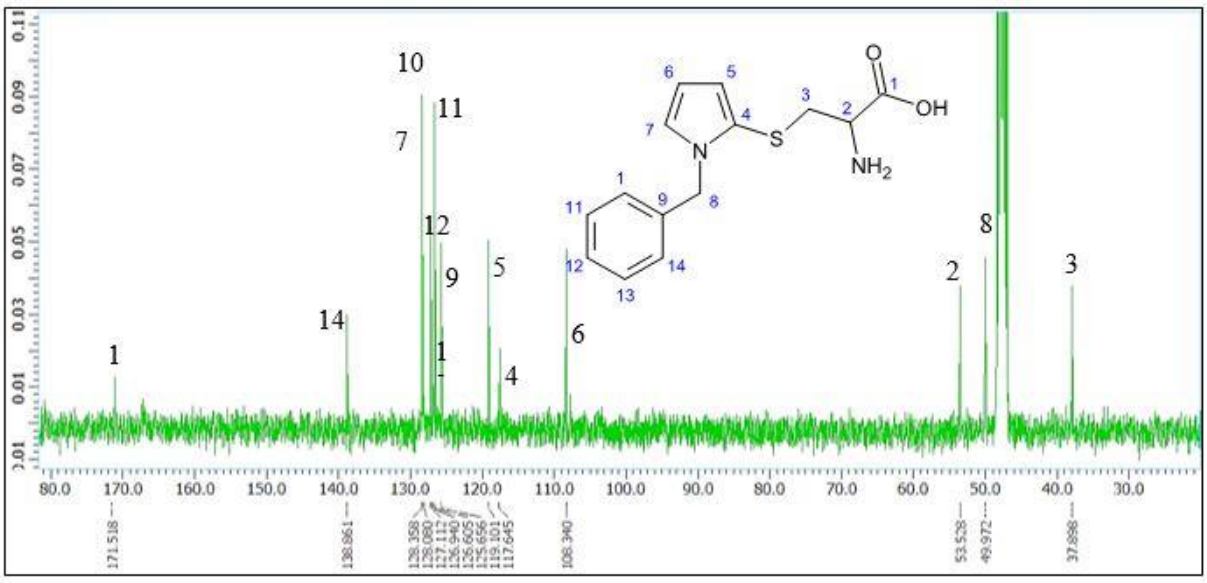


Figure 3.9 ^{13}C NMR spectra of S-(N-benzylpyrrol-2-yl)cysteine.

3.2.4 IR analysis

Concerning the bands related to IR analysis, main bands resonated at 1427, 1605, 2900, 3025, 3200, 3300 and 3606 cm^{-1}, the last of which was attributed to the functional stretching band of the hydroxyl (OH) groups. The weak bands at 3200 and 3300 cm^{-1} corresponded to the NH_2 group. The band related to the carbonyl C=O group was visible at 1605 cm^{-1} and the stretching bands at 1427 and 3025 cm^{-1} belonged to the C=C aromatic and C-H aromatic groups, respectively. The weak band at 2900 cm^{-1} was assigned to the C-H alkane group (**Figure 3.10**).

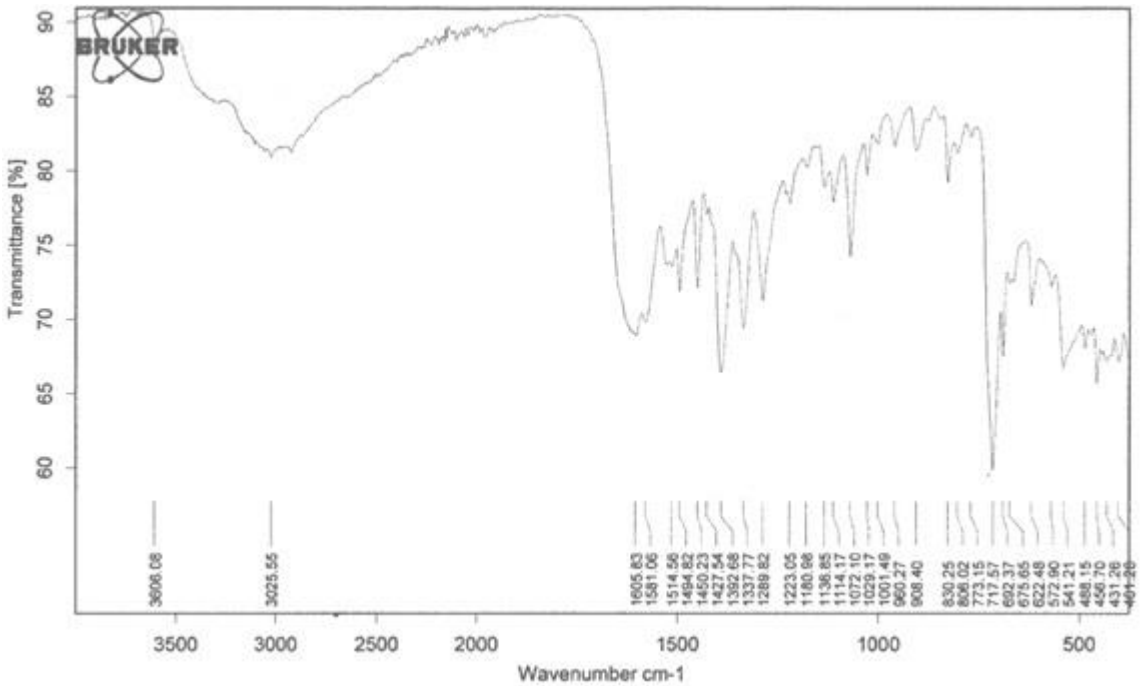


Figure 3.10 IR spectrum of S-(N-benzylpyrrol-2-yl)cysteine.

3.2.5 ESI-MS analysis

The mass spectrum related to ESI-MS analysis showed positive molecular ions at *m/z* 277 $[M+H]^+$, *m/z* 299 $[M+Na]^+$, and *m/z* 553 $[2M+H]^+$, matching the expected molecule S-(N-benzylpyrrol-2-yl)cysteine (**Figure 3.11**).

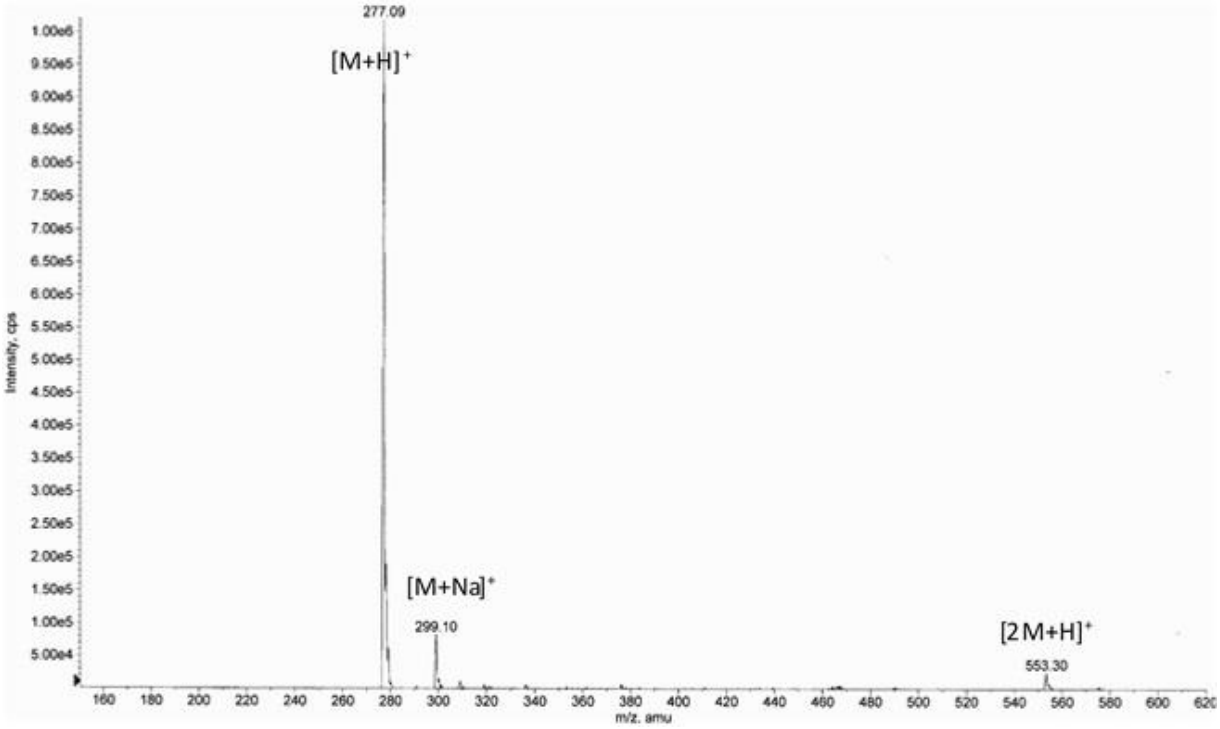
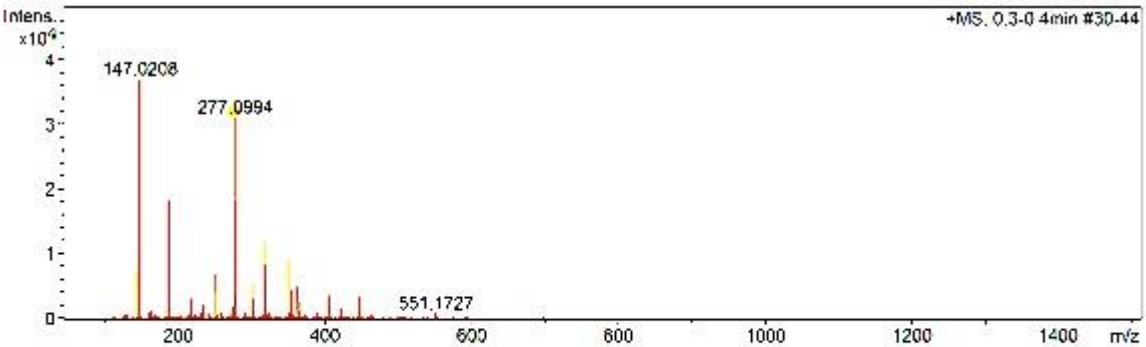


Figure 3.11 ESI-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine.

3.2.6 HR-MS analysis



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
277.0994	1	C14H17N2O2S	277.1005	4.2	1.4	1	100.00	7.5	even	ok
	2	C12H18N2NaO2S	277.0981	-4.5	10.6	2	80.63	4.5	even	ok
	3	C10H13N8S	277.0978	-5.5	11.6	3	66.15	8.5	even	ok
	4	C17H18NaS	277.1021	10.0	12.6	4	24.06	8.5	even	ok

Figure 3.12 HR-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine.

The high-resolution mass HR-MS analysis showed a peak at *m/z* 277.0994 $[M+H]^+$ indicating the existence of $C_{14}H_{17}N_2O_2S$ with a theoretical value of *m/z* 277.1005. Nevertheless, the difference of 1.1 mDa was considered negligible (**Figure 3.12**).

3.2.7 Analytical HPLC method for identification and purification of synesthetic product S-(N-benzylpyrrol-2-yl)cysteine

3.2.7.1 HPLC chromatogram

According to the HPLC chromatogram given in **Appendix 4** and **Appendix 7**, a relatively strong and distinct peak was visible at 22.99 min, which corresponds to the product S-(N-benzylpyrrol-2-yl)cysteine.

3.2.7.2 Preparative HPLC

3.2.7.2.1 HPLC chromatogram after preparative HPLC

The purification of the synthesized product with the application of preparative-HPLC is given in **Appendix 8**, in which the peak at 23.87 min characterizes the analyzed product.

3.3 Synthesis of S-(N-methylpyrrol-2-yl)cysteine

S-(N-methylpyrrol-2-yl)cysteine synthesized with a yield of 22.5% analogous to S-(2-pyrrolyl)cysteine is a worthy of attention.

3.3.1 TLC analysis

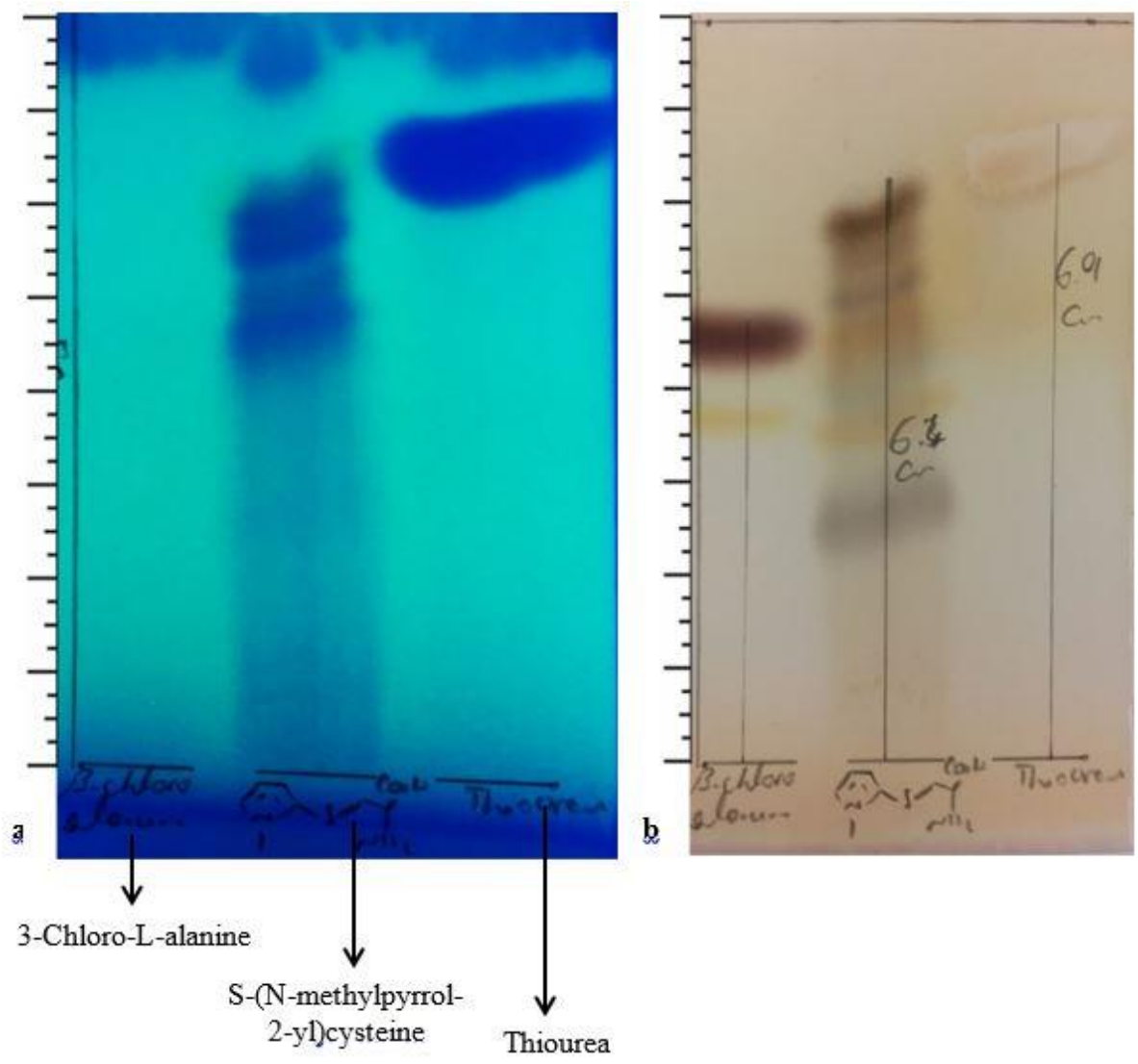


Figure 3.13 TLC analysis of S-(N-methylpyrrol-2-yl)cysteine: a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

During the TLC analysis and under a UV light, the spot related to S-(N-methylpyrrol-2-yl)cysteine was observed at Rf = 0.84 and the spot of thiourea occurred at Rf = 0.86. After spraying the plate with ninehydrin, the spot of S-(N-methylpyrrol-2-yl)cysteine appeared at Rf = 0.84, and 3-chloro-L-alanine at Rf = 0.56. In addition, 3-chloro-L-alanine was not visible at the product line indicating its full consumption (**Figure 3.13**).

3.3.2 ^{1}H-NMR analysis

Using D_2O as a solvent in H-NMR analysis, one peak was shown for the methin hydrogen group at δ = 3.78 ppm (*J* = 8.70, 3.43 Hz, dd) with integral 1. The methylene hydrogen groups 4 and 5 presented signals at δ = 3.00 ppm (*J* = 14.43, 8.70 Hz, dd) and δ = 3.66 ppm (*J* = 14.43, 5.04 Hz, dd), respectively, each with integral 1. The methyl hydrogens linked to nitrogen showed a chemical shift at δ = 3.76 ppm; this was a singlet with integral 3. Hydrogens 1′, 2′, and 3′ belonging to the pyrrole group each with integral 1 were observed at δ = 6.20 ppm (md), δ = 6.51 ppm (md), and δ = 7.02 ppm (md), respectively (**Table 3.1(a)** and **Figure 3.14**).

3.3.3 ^{13}C-NMR analysis

^{13}C-NMR analysis of S-(N-methylpyrrol-2-yl)cysteine using D_2O as the analysis solvent was not straightforward because of its high instability and unexpected peaks. The carboxylic carbon showed a typical chemical shift at δ = 172.5 ppm for the aliphatic part. Moreover, the methin and methylene groups were observed at δ = 53.74 ppm and δ = 36.92 ppm, respectively. In the heteroaromatic part, carbons 4, 5, 6, and 7 corresponding to the pyrrole group showed sharp signals at δ = 117.91 ppm, δ = 118.43 ppm, δ = 108.32 ppm and δ = 127.29 ppm, respectively (**Table 3.1(b)** and **Figure 3.15**).

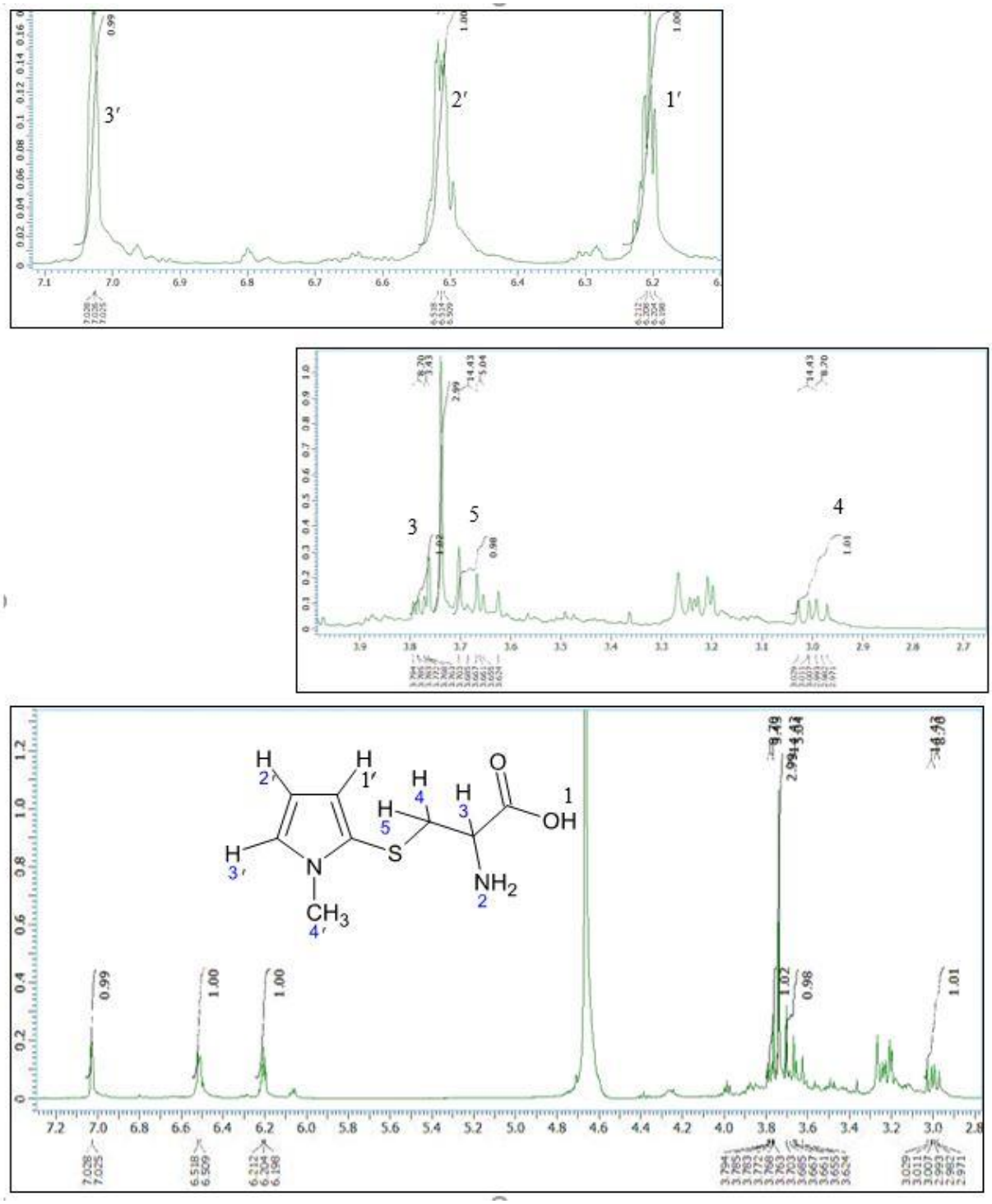


Figure 3.14 ^{1}H NMR spectra of S-(N-methylpyrrol-2-yl)cysteine.

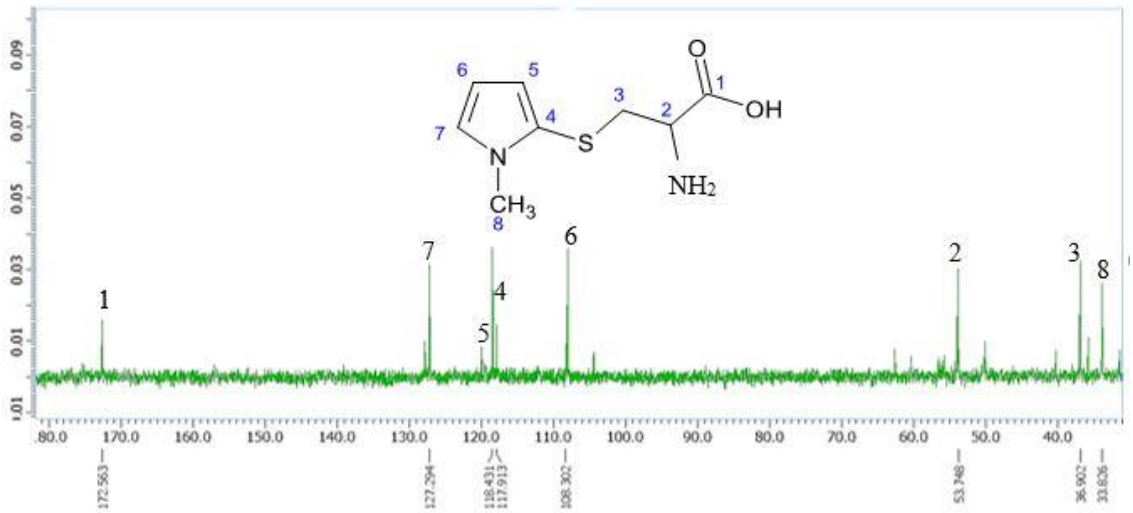


Figure 3.15 ^{13}C NMR spectra of S-(N-methylpyrrol-2-yl)cysteine.

3.3.4 IR analysis

Diverse stretching bands relevant to the IR analysis appeared at 1427, 1614, 2950, 3000, 3156, 3214 and 3242 cm^{-1}, the strongest of which was visible at 1614 cm^{-1-}, which corresponds to the carbonyl C=O group. The bands at 3156 and 3214 cm^{-1} belonged to the NH_2 group. The stretching bands at 1457 and 3000 cm^{-1} referred to the C=C aromatic and C-H aromatic groups, respectively. The stretching bands at 3000 and 3242 cm^{-1} were attributed to the C-H alkane and hydroxyl (OH) groups, respectively (**Figure 3.16**).

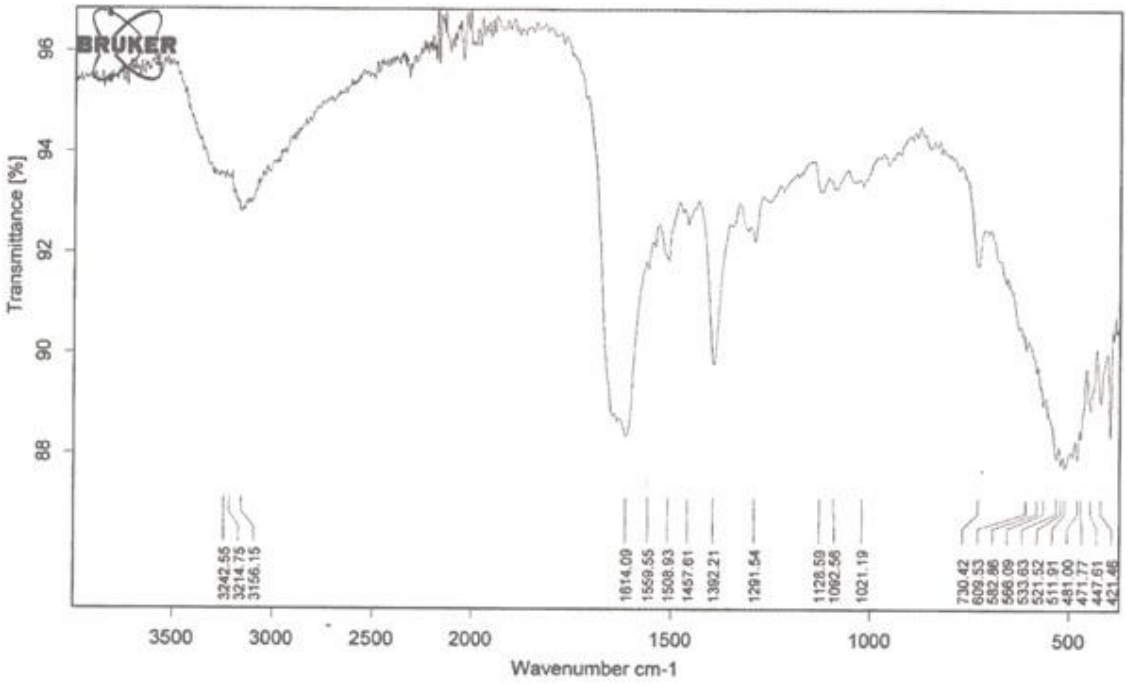


Figure 3.16 IR spectrum of S-(N-methylpyrrol-2-yl)cysteine**.**

3.3.5 ESI-MS analysis

The mass spectrum in ESI-MS analysis showed positive molecular ions at *m/z* 201 $[M+H]^+$, *m/z* 223 $[M+Na]^+$ and *m/z* 239 $[M+K]^+$confirming the expected molecule S-(N-methylpyrrol-2-yl)cysteine (**Figure 3.17**).

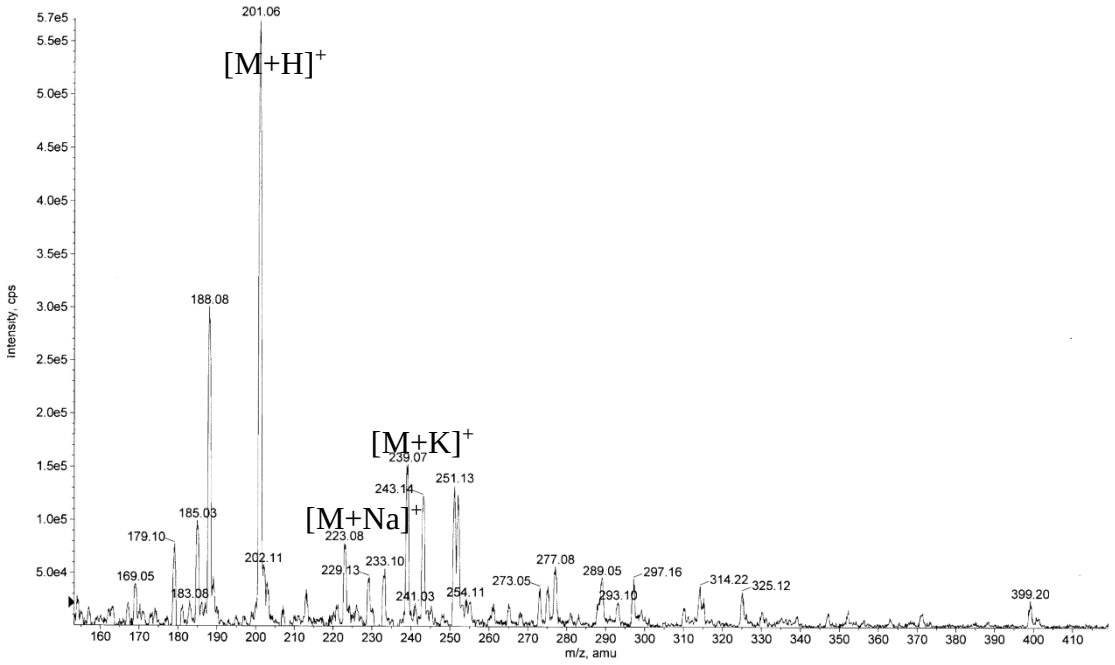


Figure 3.17 ESI-MS analysis of S-(N-methylpyrrol-2-yl)cysteine.

3.3.6 HR-MS analysis

The high-resolution mass spectrum consistent with the molecular formula $C_8H_{13}N_2O_2S$ showed a signal at *m/z* 201.0692 $[M+H]^+$ with a difference of 0.5 mDa in comparison to the one measured at *m/z* 201.0687 $[M+H]^+$), which was not significant (**Figure 3.18**).

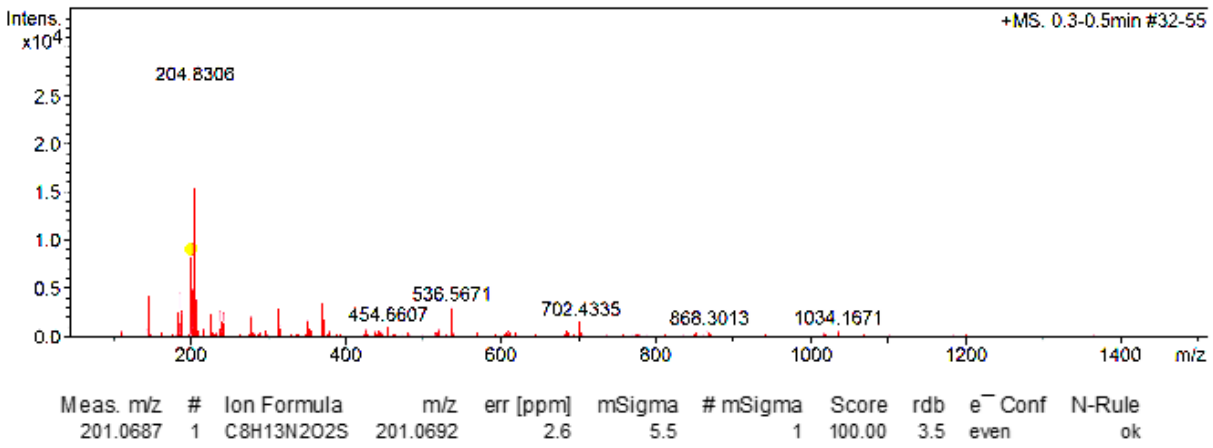


Figure 3.18 HR-MS analysis of S-(N-methylpyrrol-2-yl)cysteine.

3.4 Synthesis of S-(2-pyrrolyl)cysteine

Synthesis of S-(2-pyrrolyl)cysteine with a yield of 35% was the desired product leading to the formation of S-(2-pyrrolyl)cysteine S-oxide after oxidation.

3.4.1 TLC analysis

Following a TLC analysis, one spot indicating S-(2-pyrrolyl)cysteine was observed under UV light (**Figure 3.19**) after the plate had been sprayed by ninhydrin at Rf = 0.63. Although the lack of 3-chloro-L-alanine in the product line (Rf = 0.39) represented a fully progressive reaction, thiourea was still visible (Rf = 0.81).

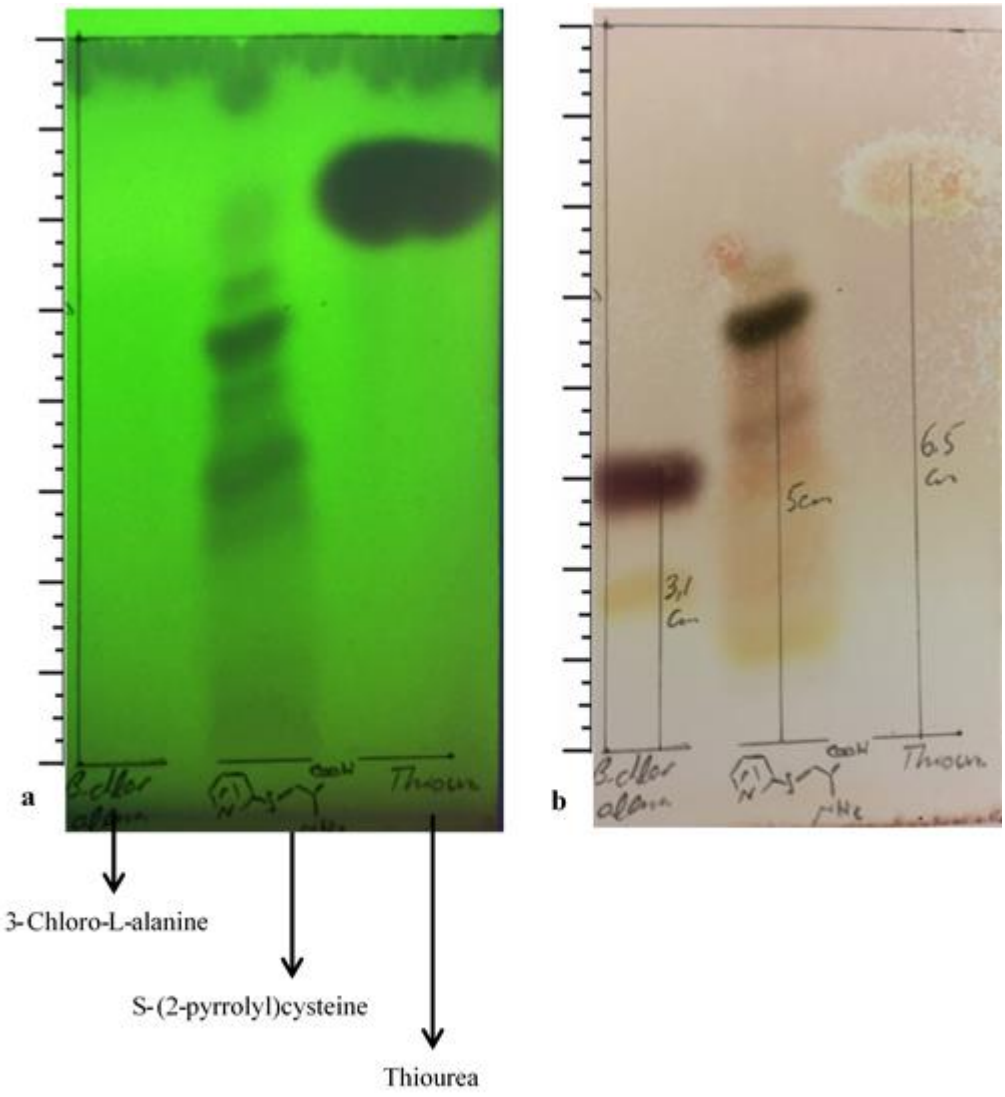


Figure 3.19 Analysis by TLC of S-(2-pyrrolyl)cysteine. a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

3.4.2 ^{1}H-NMR analysis

In order to perform the H-NMR analysis, we used D_2O since this compound is readily water-soluble. The methin hydrogen in the aliphatic part with integral 1 was observed at δ = 3.85 ppm (*J* = 3.89, 8.70, Hz, dd). Additionally, the methylene hydrogens 4 and 5, each with integral 1, appeared at δ = 3.11 ppm (*J* = 8.70, 14.65 Hz, dd) and δ = 3.36 ppm (*J* = 3.66, 14.65 Hz, dd), respectively.

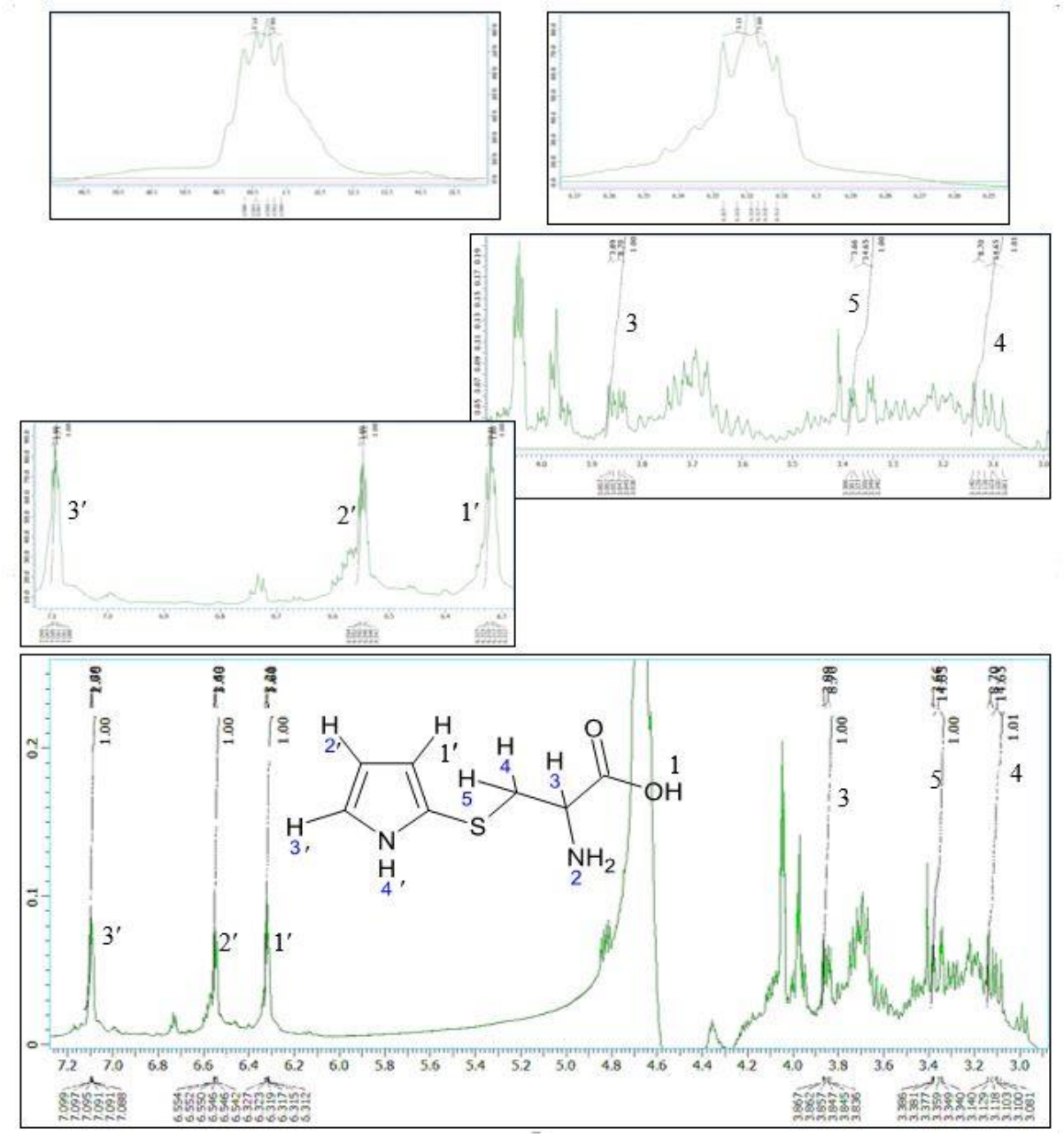


Figure 3.20 ^{1}H NMR spectra of S-(2-pyrrolyl)cysteine.

In the aromatic part of the molecule, hydrogens 1′, 2′ and 3′ corresponding to the pyrrole group, each with integral 1, were shown at δ = 6.32 ppm, (J = 3.21, 1.60 Hz, dd), δ = 6.55 ppm (J = 1.60, 3.43 Hz, dd), and δ = 7.09 ppm (J = 1.60, 2.75 Hz, dd), respectively. Other unknown signals can be considered by-products due to the instability of the S-(2-pyrrolyl)cysteine in the presence of light, oxygen, and room temperature. **Table 3.1(a)** and **Figure 3.20** illustrate the analysis results related to this compound.

3.4.3 ^{13}C-NMR analysis

The solvent used for C-NMR analysis was D_2O because of its high water-solubility. In this analysis, S-(2-pyrrolyl)cysteine appeared as the most unstable compound among all the synthesized products. As a result, seven peaks were observed for this compound. In addition, the carboxylic carbon demonstrated a typical chemical shift at δ = 172.57 ppm. The carbons of the aliphatic methin and methylene groups were distinguished at δ = 54.15 ppm and δ = 34.85 ppm, respectively. The carbons 4, 5, 6, and 7 in the aromatic part, corresponding to the pyrrole group, displayed chemical shifts at δ = 123 ppm, δ = 117.37 ppm, δ = 109.62 ppm, and δ = 119.65 ppm, respectively (**Table 3.1(b)** and **Figure 3.21**).

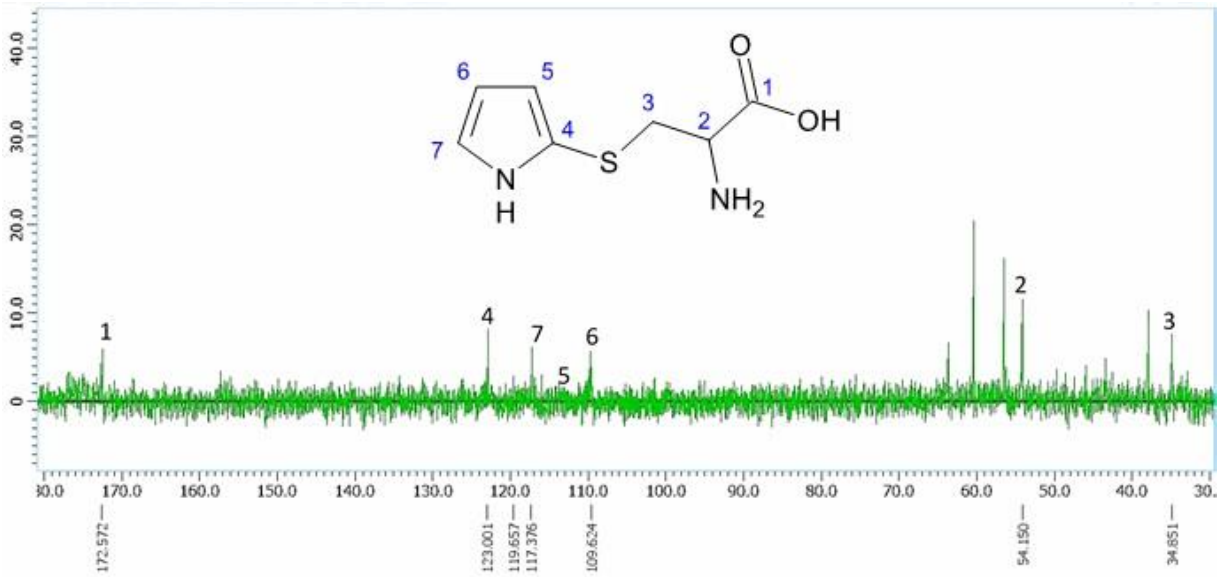


Figure 3.21 ^{13}C NMR spectra of S-(2-pyrrolyl)cysteine.

3.4.4 IR analysis

In this analysis, the stretching bands emerged at 1388, 1608, 2900, 3000, 3134, and 3300 cm^{-1}. The two last ones belonged to the OH and NH_2 groups, respectively. Additionally, the strong and weak bands corresponding to the carbonyl (C=O) and C-H alkane groups were visible at 1608 and 2900 cm^{-1}, respectively. The weak bands of 1388 and 3000 cm^{-1} were related to the C=C and C-H aromatic groups, respectively (**Figure 3.22**).

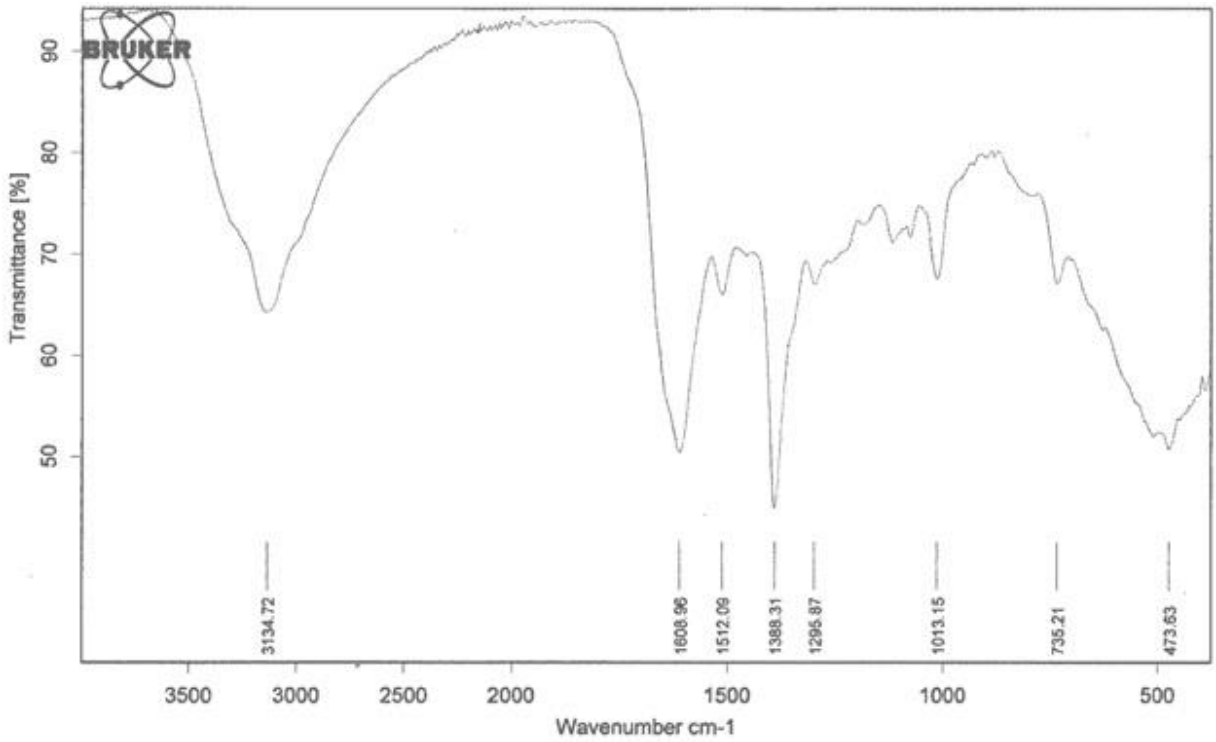


Figure 3.22 IR spectrum of S-(2-pyrrolyl)cysteine.

3.4.5 ESI-MS analysis

Figure 3.23 shows the mass spectra at *m/z* 187 $[M+H]^+$ and *m/z* 209 $[M+Na]^+$, confirming S-(2-pyrrolyl)cysteine as the end product.

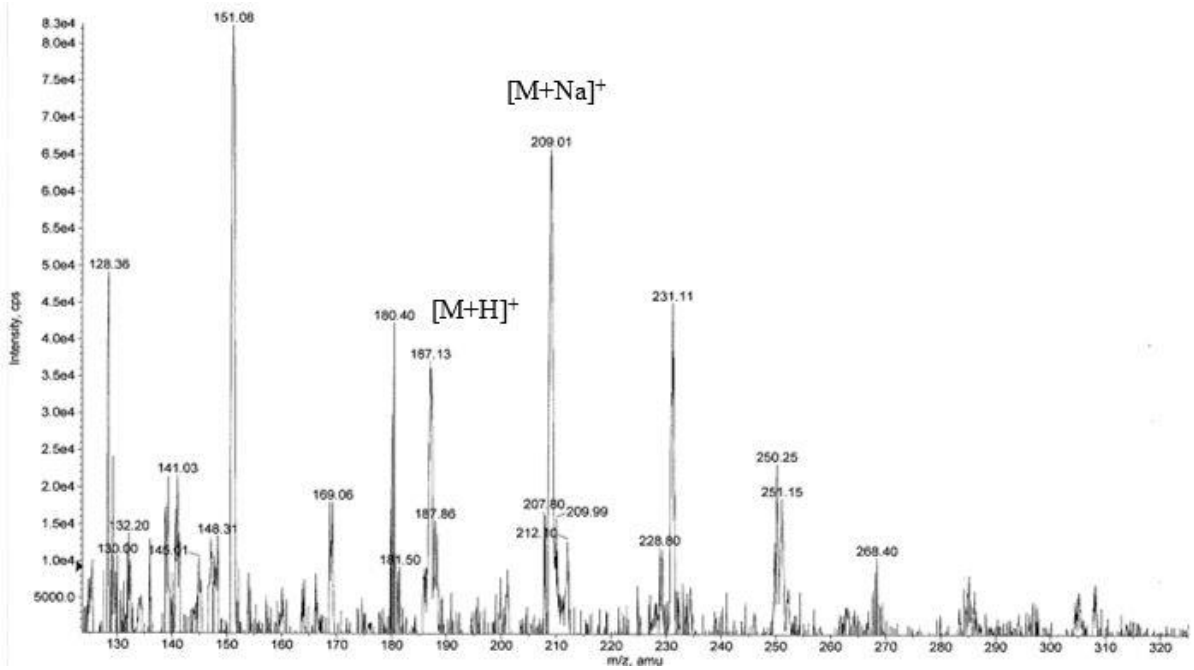
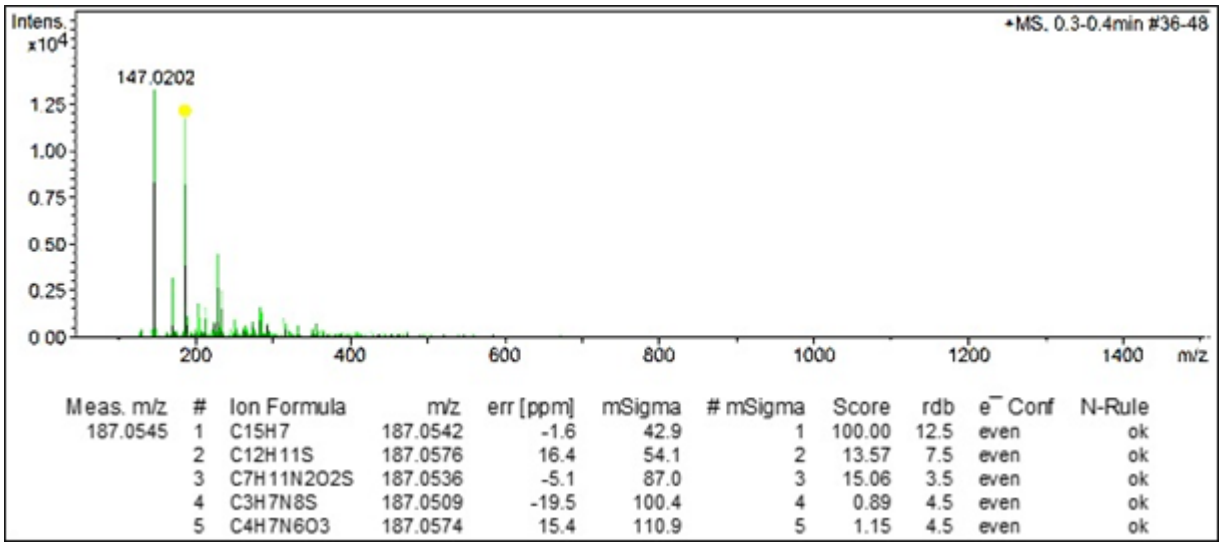


Figure 3.23 MS analysis of S-(2-pyrrolyl)cysteine.

3.4.6 HR-MS analysis

The ratio of molecular weight to molecular charge measured through HR-MS analysis was *m/z* 187.0545 $[M+H]^+$. It corresponds to the molecular formula of $C_7H_{11}N_2O_2S$ and is consistent with the theoretical *m/z* ratio of 187.0536 and an acceptable difference in value of 0.9 mDa (**Figure 3.24**).



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
187.0545	1	C15H7	187.0542	-1.6	42.9	1	100.00	12.5	even	ok
	2	C12H11S	187.0576	16.4	54.1	2	13.57	7.5	even	ok
	3	C7H11N2O2S	187.0536	-5.1	87.0	3	15.06	3.5	even	ok
	4	C3H7N8S	187.0509	-19.5	100.4	4	0.89	4.5	even	ok
	5	C4H7N6O3	187.0574	15.4	110.9	5	1.15	4.5	even	ok

Figure 3.24 HR-MS analysis of S-(2-pyrrolyl)cysteine.

3.5 Synthesis of S-(N-benzylpyrrol-2-yl)cysteine S-oxide

S-(N-benzylpyrrol-2-yl)cysteine S-oxide was synthesized with a yield of 30% after purification using preparative HPLC.

3.5.1 TLC analysis

Using thin-layer chromatography, the possible existence of S-(N-benzylpyrrol-2-yl)cysteine S-oxide was first confirmed and then detected using ninhydrin reagent and UV light. The lack of any spot at Rf = 0.737 can be interpreted as the full oxidization of the primary product. The calculated Rf for the product was 0.5 after ninhydrin spraying (**Figure 3.25**).

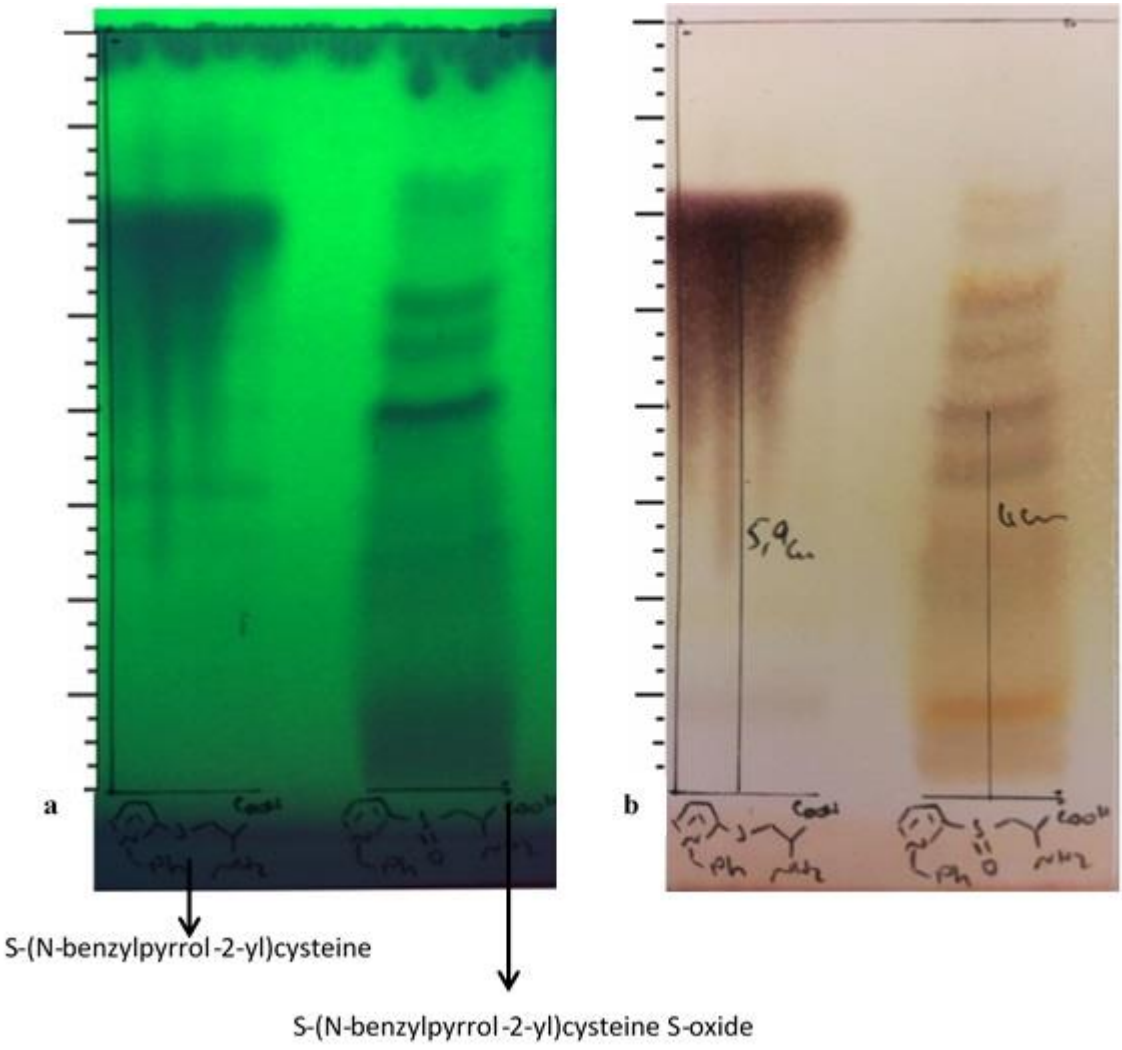


Figure 3.25 TLC analysis of S-(N-benzylpyrrol-2-yl)cysteine S-oxide: a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

3.5.2 ^{1}H-NMR analysis

The solvent used in this analysis was D_2O.

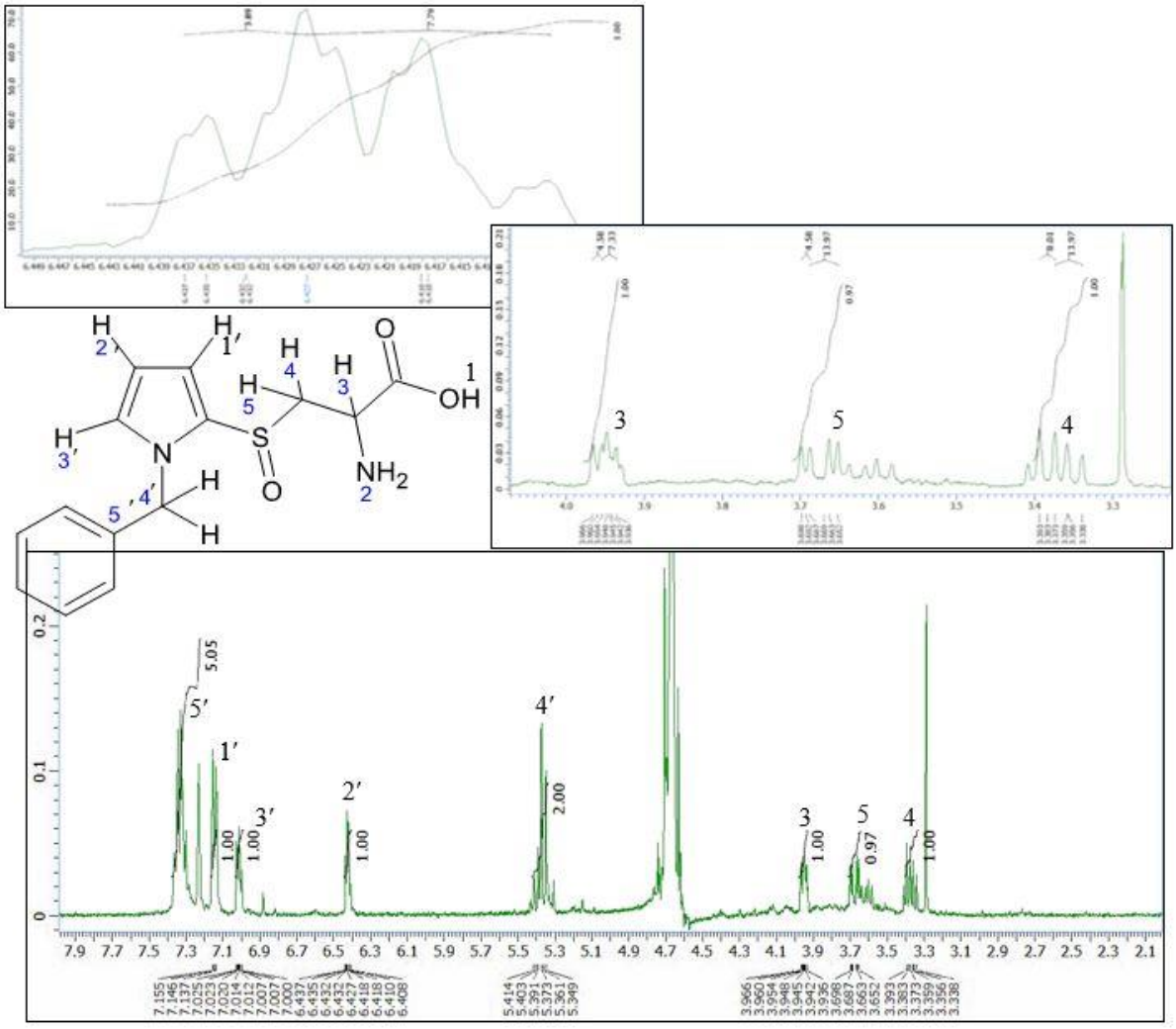


Figure 3.26 ^{1}H NMR spectra of S-(N-benzylpyrrol-2-yl)cysteine S-oxide.

According to the results, the hydrogens H1-H5 related to the aliphatic part, each with integral 1, appeared as follows: H3 at δ = 3.95 ppm (*J* = 4.58, 7.33 Hz, dd), H4 at δ = 3.36 ppm (*J* = 8.00, 13.97 Hz, dd), and H5 at δ = 3.67 ppm (*J* = 4.50, 13.97 Hz, dd). In the heteroaromatic part of the compound, hydrogens 1′, 2′ and 3′ belonging to the pyrrole ring, each with integral 1, were observed at δ = 7.14 ppm(md), δ = 16.42 ppm (md), and δ = 7.04 ppm (*J* = 7.33 Hz, dd), respectively. Hydrogens 4′ of the benzyl group with integral 2 were observed at δ = 5.37 ppm (md) while hydrogens 5′ of the aromatic part with integral 5 showed a peak at δ = 7.3 ppm (md) (**Table 3.1(a)** and **Figure 3.26**).

3.5.3 ^{13}C-NMR analysis

D_2O was used in the ^{13}C-NMR analysis of the S-(N-benzylpyrrol-2-yl)cysteine S-oxide. The analysis demonstrated a typical chemical shift at δ = 171.90 ppm for the carboxylic carbon 1. As shown in **Table 3.1(b)**, methin carbon and methylene carbon were identified at δ = 47.20 ppm and at δ = 56.30 ppm, respectively. Additionally, the carbon of the benzylic group was visible at δ = 49.32 ppm. In the hetero aromatic part, the carbons 4–7 of the pyrrole group with low density were recognized at δ = 137.49 ppm, δ = 115.22 ppm, δ = 105.88 ppm, and δ = 120.69 ppm, respectively. In the phenyl ring, carbons 9–14 with chemical shifts at 127.00, 128.80, 127.50, 129.30, 127.10, and 135.00 ppm were distinguished (**Table 3.1(b)** and **Figure 3.27**).

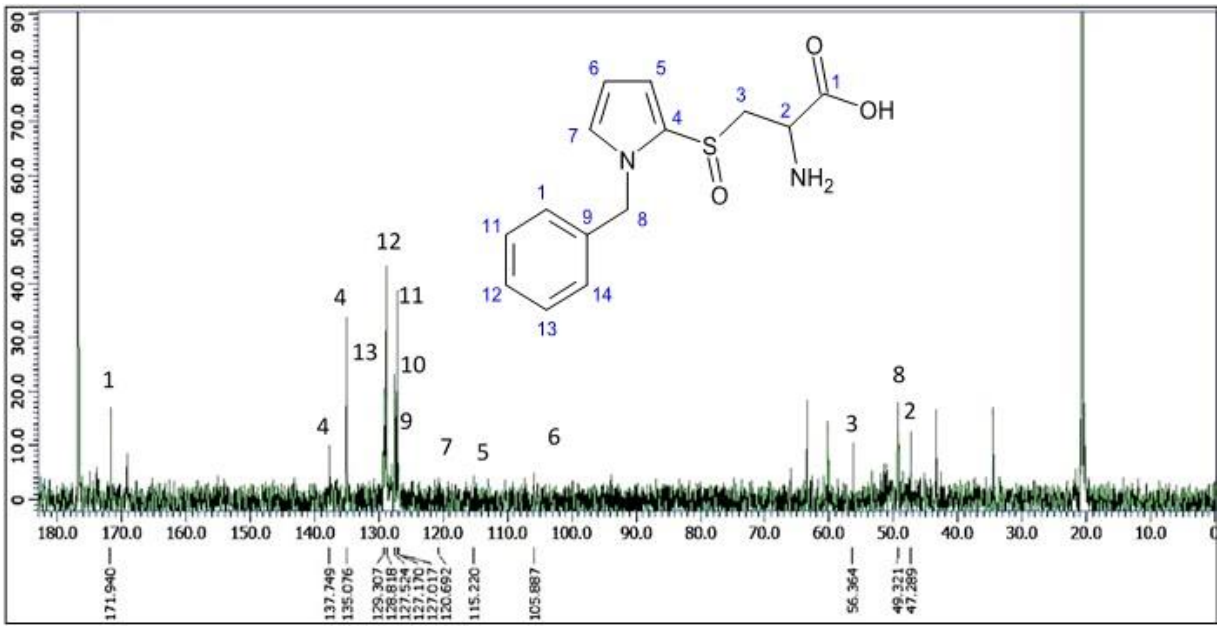


Figure 3.27 ^{13}C NMR of S-(N-benzylpyrrol-2-yl)cysteine S-oxide.

3.5.4 IR analysis

The stretching bands resulting from this analysis were observed at 1240, 1496, 1706, 2603, 2900, 2927, 3100, and 3400 cm^{-1}. The band at 1240 cm^{-1} represented the S=O group. The bands at 1706 and 3400 cm^{-1} were representative of the C=O and OH groups. The weak bands at 2927 and 3100 cm^{-1} were related to the NH_2 group, and the stretching bands corresponding to the aromatic C=C and C-H groups were visible at 1496 and 2900 cm^{-1}, respectively. Additionally, the weak band at 2603 cm^{-1} was associated with the C-H alkane group (**Figure 3.28**).

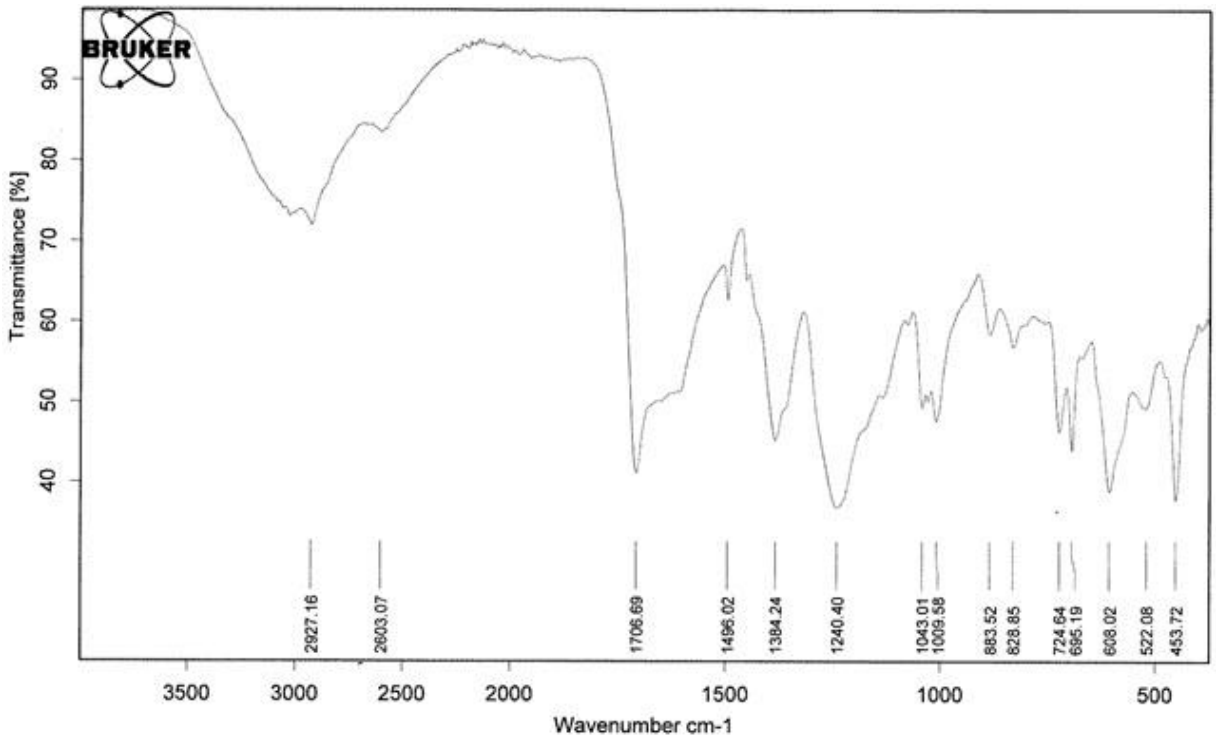


Figure 3.28 IR spectrum of S-(N-benzylpyrrol-2-yl)cysteine S-oxide.

3.5.5 ESI-MS analysis

The ESI-MS analysis revealed a basic remarkable peak related to the compound S-(N-benzylpyrrol-2-yl)cysteine S-oxide at *m/z* ratio of 293 $[M+H]^+$. In addition, the peaks *m/z* 315 $[M+Na]^+$ and *m/z* 331 $[M+K]^+$ confirmed the formation of the desired product (**Figure 3.29**).

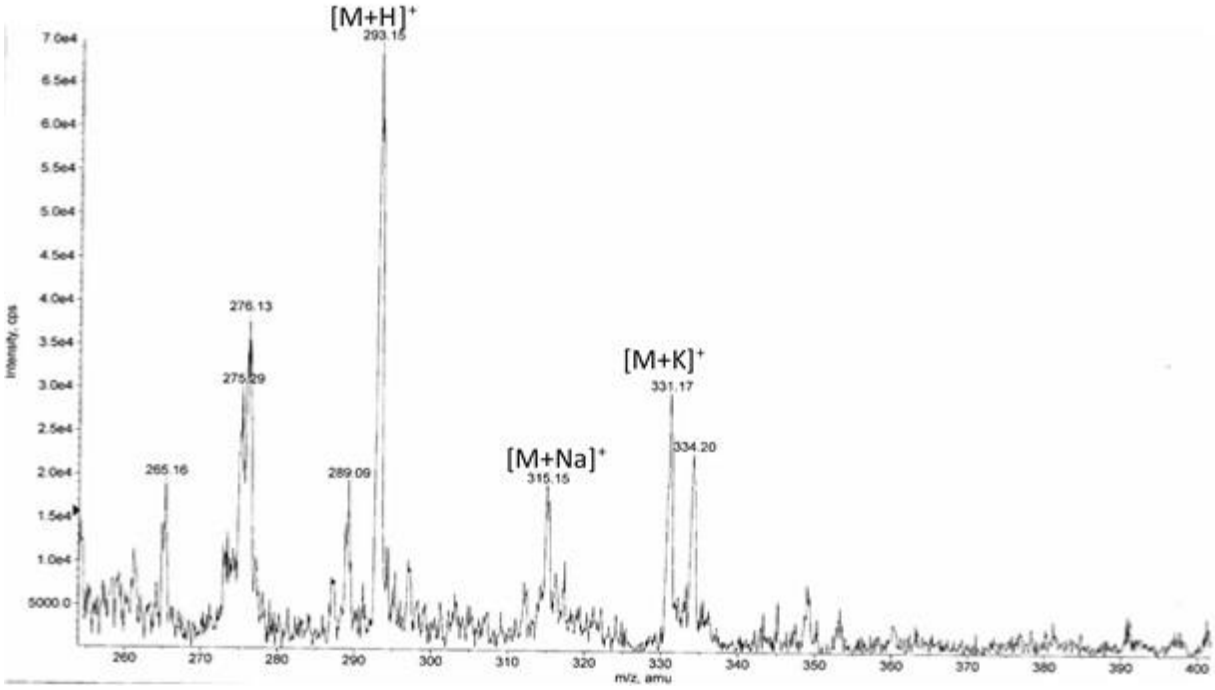


Figure 3.29 MS analysis of S-(N-benzylpyrrol-2-yl)cysteine S-oxide.

3.5.6 Analytical HPLC method for identification and purification of synesthetic product S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

3.5.6.1 HPLC chromatogram

The results of the HPLC analysis of S-(N-benzylpyrrol-2-yl)cysteine S-oxide, supported by HPLC-MS analysis, are shown in **Appendix 9**, in which the peak at 23.8 min describes the intended product S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (**Appendix 5**).

3.5.6.2 ESI-MS analysis

Appendix 10 contains the results of the ESI-MS analysis before the preparative-HPLC. The ions at *m/z* 277 $[M+H]^+$ and *m/z* 293 $[M+H]^+$ corresponded to S-(N-benzylpyrrol-2-yl)cysteine and S-(N-benzylpyrrol-2-yl)cysteine S-oxide, respectively.

3.5.6.3 HPLC-MS analysis

The HPLC-MS analysis revealed a visible peak at 28.57 min with *m/z* 293 $[M+H]^+$ corresponding to the oxidized form of the compound S-(N-benzylpyrrol-2-yl)cysteine. The peak at 33.98 min with *m/z* 277 $[M+H]^+$ correlated to S-(N-benzylpyrrol-2-yl)cysteine (Appendices 11–12). Preparative-HPLC was used to separate and purify these compounds.

3.5.6.4 Preparative HPLC

The HPLC chromatogram illustrated in **Appendix 13** shows the peak of the purified product at 5.46 min corresponding to S-(N-benzylpyrrol-2-yl)cysteine S-oxide.

3.5.6.4.1 ***ESI-MS analysis after preparative-HPLC***

Regarding the ESI-MS analysis, various peaks of the purified product S-(N-benzylpyrrol-2-yl)cysteine S-oxide at *m/z* 293 $[M+H]^+$, *m/z* 315 $[M+Na]^+$, *m/z* 331 $[M+K]^+$, *m/z* 585 $[2M+H]^+$, *m/z* 607 $[2M+Na]^+$, and *m/z* 899 $[3M+Na]^+$ confirmed the existence of the desired product (**Appendix 14**).

3.6 Synthesis of S-(2-thienyl)cysteine

The synthesis of S-(2-thienyl)cysteine was obtained with a yield of 93%.

3.6.1 TLC analysis

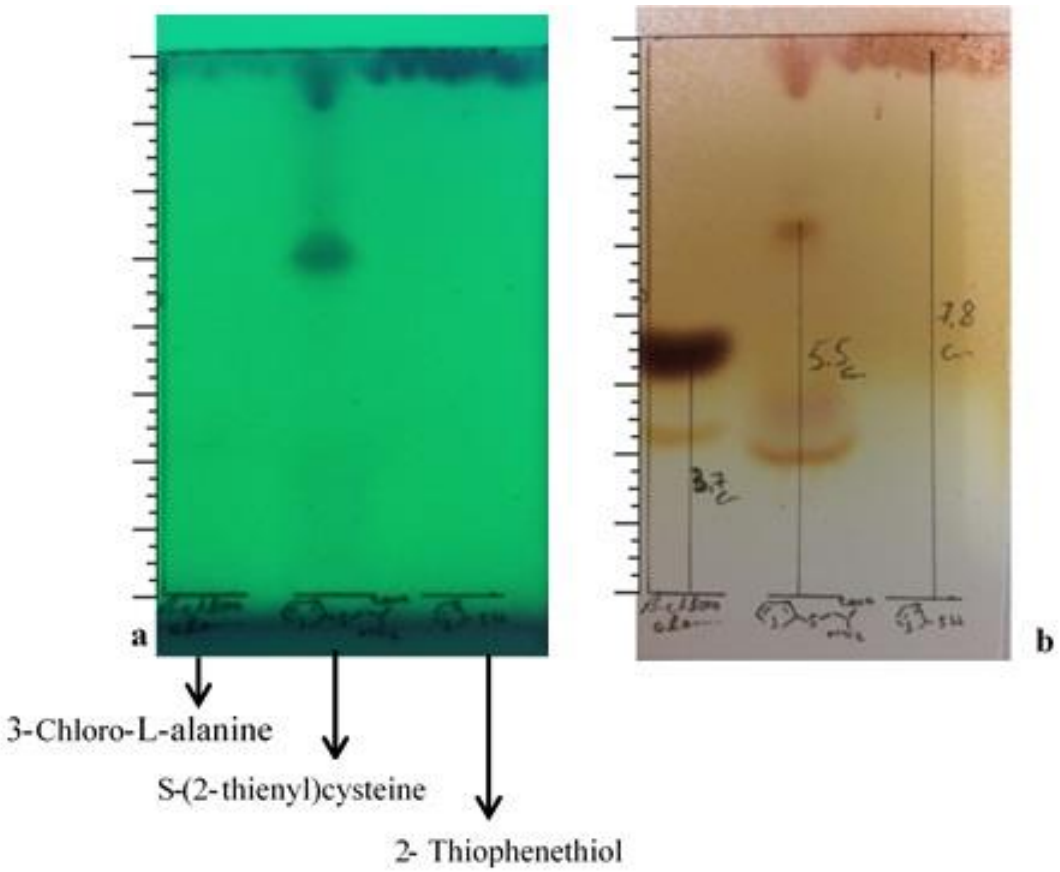


Figure 3.30 TLC analysis by of S-(2-thienyl)cysteine: a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

In this analysis, 3-chloro-L-alanine (Rf = 0.462) and 2-thiophene thiol (Rf = 0.98) were used as reference. After it was sprayed with ninhydrin, the S-(2-thienyl)cysteine stained at Rf = 0.69 and the 3-chloro-alanine was no longer visible in the product line (**Figure 3.30**).

3.6.2 ^{1}H-NMR analysis

Using D_2O as solvent for this analysis, the signals 1′–3′ were attributed to the heteroaromatic thiophene ring and the signals H1–H5 were allocated to the aliphatic amino acid, all with integral 1: H3 at δ = 3.75 (*J* = 8.70, 3.89 Hz, dd), H4 at δ = 3.15 (*J* = 14.65, 8.70 Hz, dd), H5 at δ = 3.4 (*J* = 14.65, 3.89 Hz, dd), H2′ at δ = 7.30 (*J* = 3.66, 1.37 Hz, dd), H1′ at δ = 7.07 (*J* = 5.27, 3.66 Hz, dd), and H3′ at δ = 7.56 (*J* = 5.27, 1.37 Hz, dd) (**Table 3.1(a)** and **Figure 3.31**).

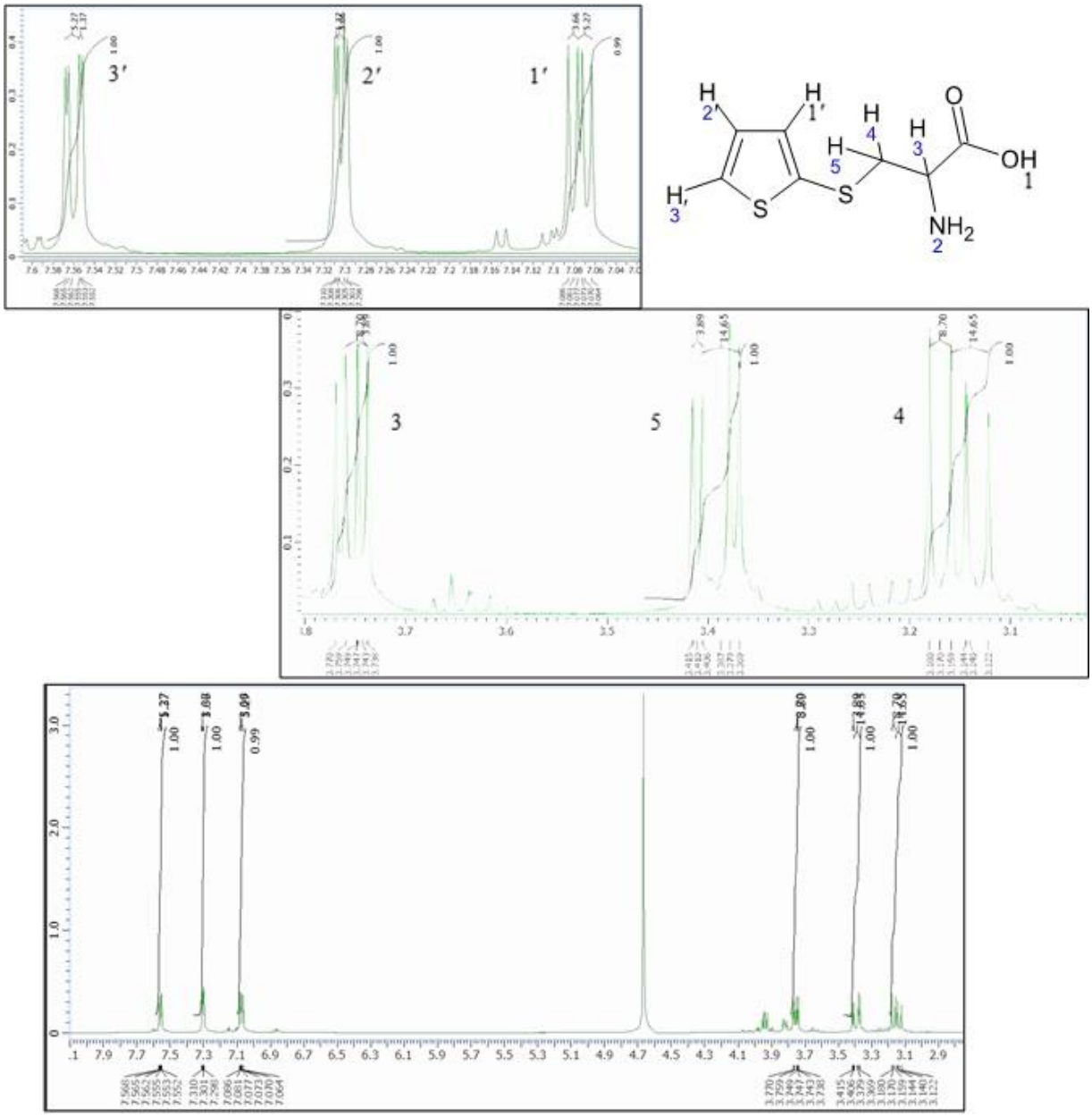


Figure 3.31 ^{1}H NMR spectra of S-(2-thienyl)cysteine.

3.6.3 ^{13}C-NMR analysis

The solvent used to conduct the C-NMR analysis was D_2O. The carboxylic carbon was observed at δ = 173.12 ppm, the methin carbon 2 at δ = 53.84 ppm, and the methylene carbon 3 at δ = 30.58 ppm. Moreover, carbons 4-7 in the aromatic part of the thiophene group caused the chemical shifts at δ = 135.6 ppm, δ = 128.42 ppm, δ = 131.42 ppm, and δ = 130.90 ppm, respectively (**Table 3.1(b)** and **Figure 3.32**).

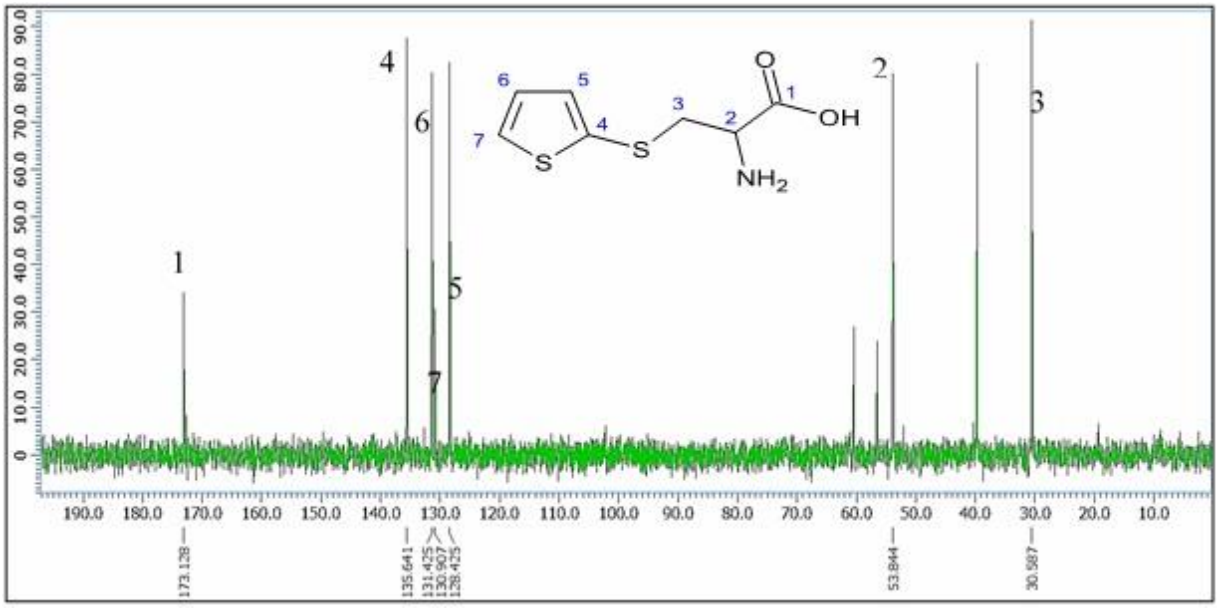


Figure 3.32 ^{13}C NMR spectra of S-(2-thienyl)cysteine.

3.6.4 IR analysis

The stretching bands resulting from the IR analysis were observed at 1510, 1581, 2600, 2950, 2977, 3100, and 3450 cm^{-1}. The most intense band at 1581 cm^{-1} belonged to the C=O group. The weak bands at 2977 and 3100 cm^{-1} corresponded to the NH_2 group and the stretching bands related to the aromatic C=C and C-H groups were observed at 1510 and 2950 cm^{-1}, respectively. Additionally, the bands at 2600 and 3450 cm^{-1} represented the C-H alkane and OH groups, respectively (**Figure 3.33**).

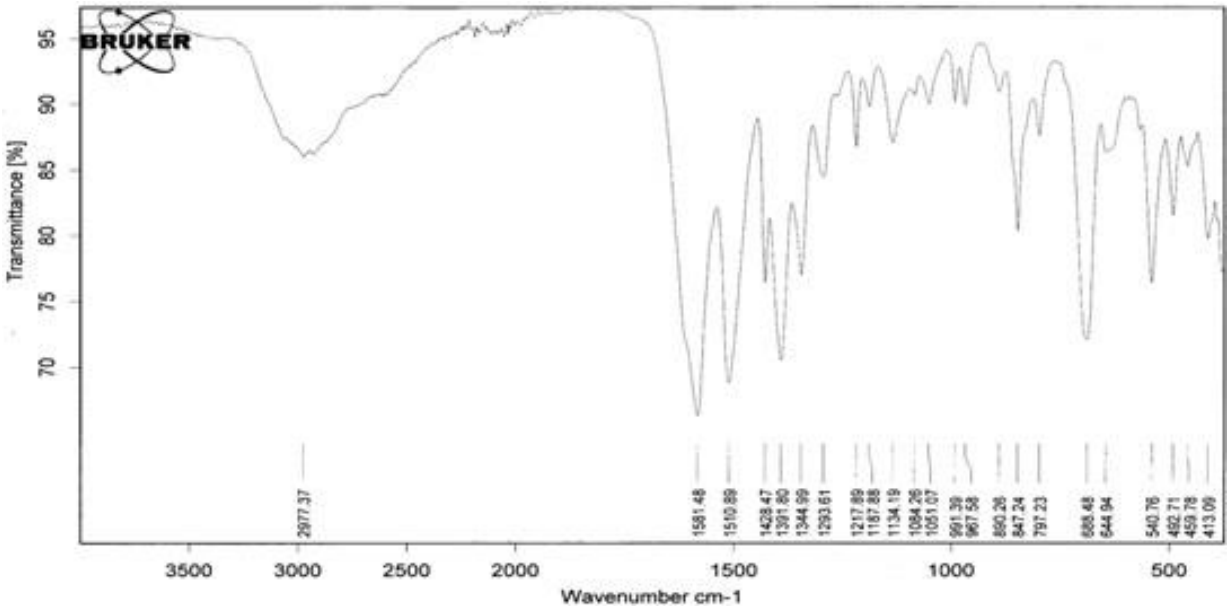


Figure 3.33 IR spectrum of S-(2-thienyl)cysteine**.**

3.6.5 ESI-MS analysis

The positive ion peaks related to the compound S-(2-thienyl)cysteine were formed in the mass spectrum of ESI-MS analysis at m/z 204.08 [M+H]+ and m/z 242 [M+K]+ (**Figure 3.34**).

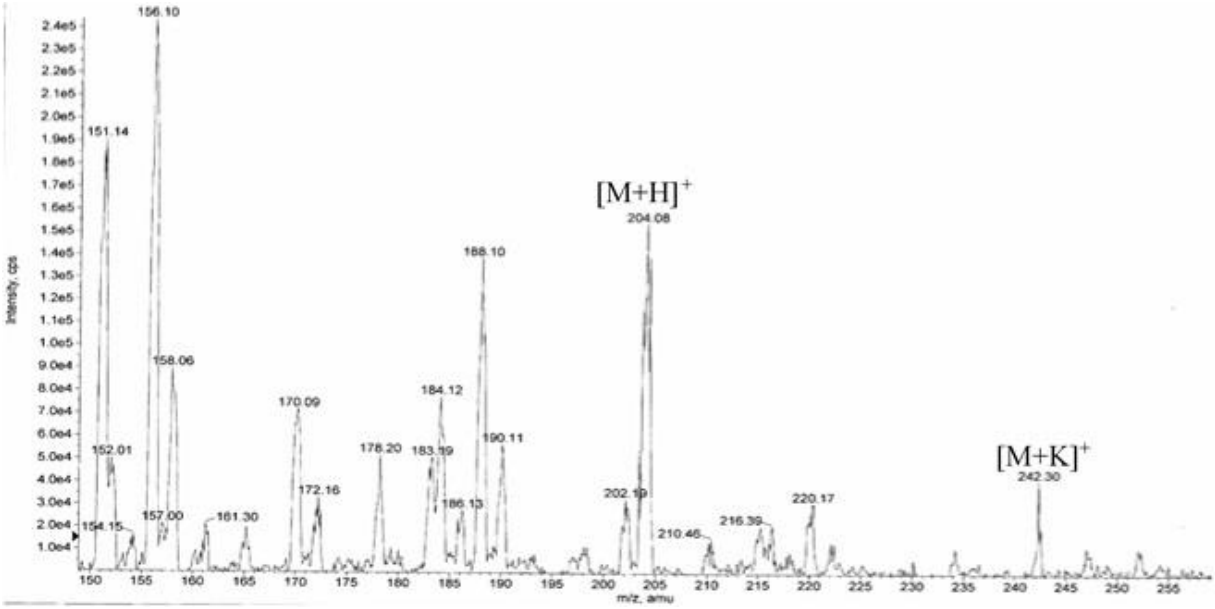
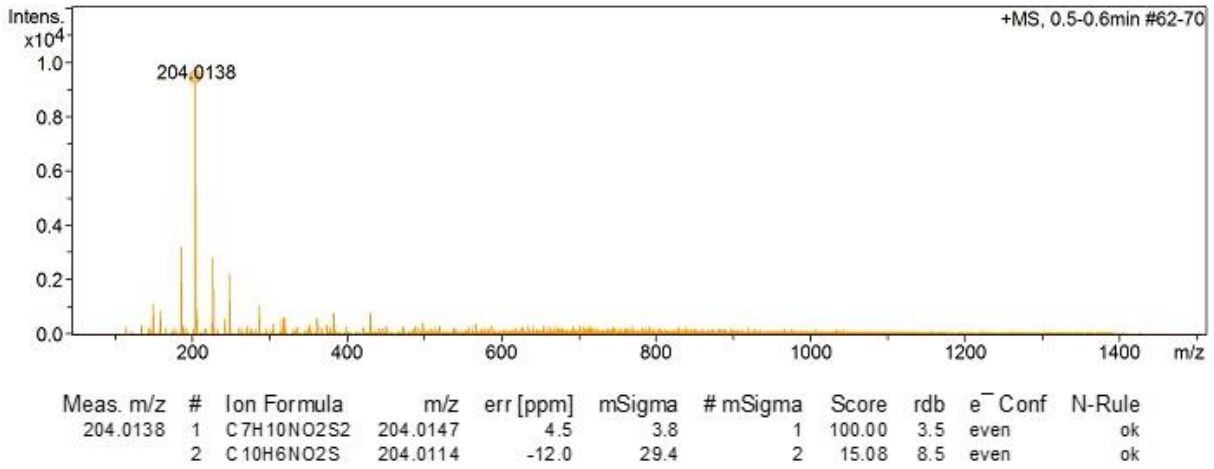


Figure 3.34 MS analysis of S-(2-thienyl)cysteine

3.6.6 HR-MS analysis



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
204.0138	1	C7H10NO2S2	204.0147	4.5	3.8	1	100.00	3.5	even	ok
	2	C10H6NO2S	204.0114	-12.0	29.4	2	15.08	8.5	even	ok

Figure 3.35 HR-MS analysis of S-(2-thienyl)cysteine.

Based on the results of the HR-MS analysis, the ratio of molecular weight to molecular charge was *m/z* 204.0138, which correlated to the molecular formula of $C_7H_{10}NO_2S_2$ with the value of *m/z* 204.0147 and an acceptable difference of about 0.9 mDa (**Figure 3.35**).

3.7 Synthesis of S-(2-thienyl)cysteine-S-oxide

3.7.1 TLC analysis

The TLC plate displays 3 different spots related to the desired product S-(2-thienyl)cysteine-S-oxide (47.3%) at Rf = 0.81, the sulfonic product (48.9%) at Rf = 0.73, and the non-oxidized product (3.7%) at Rf = 0.89. The volume of spots was measured by the TLC plate (**Figure 3.36** and **Appendix 15**).

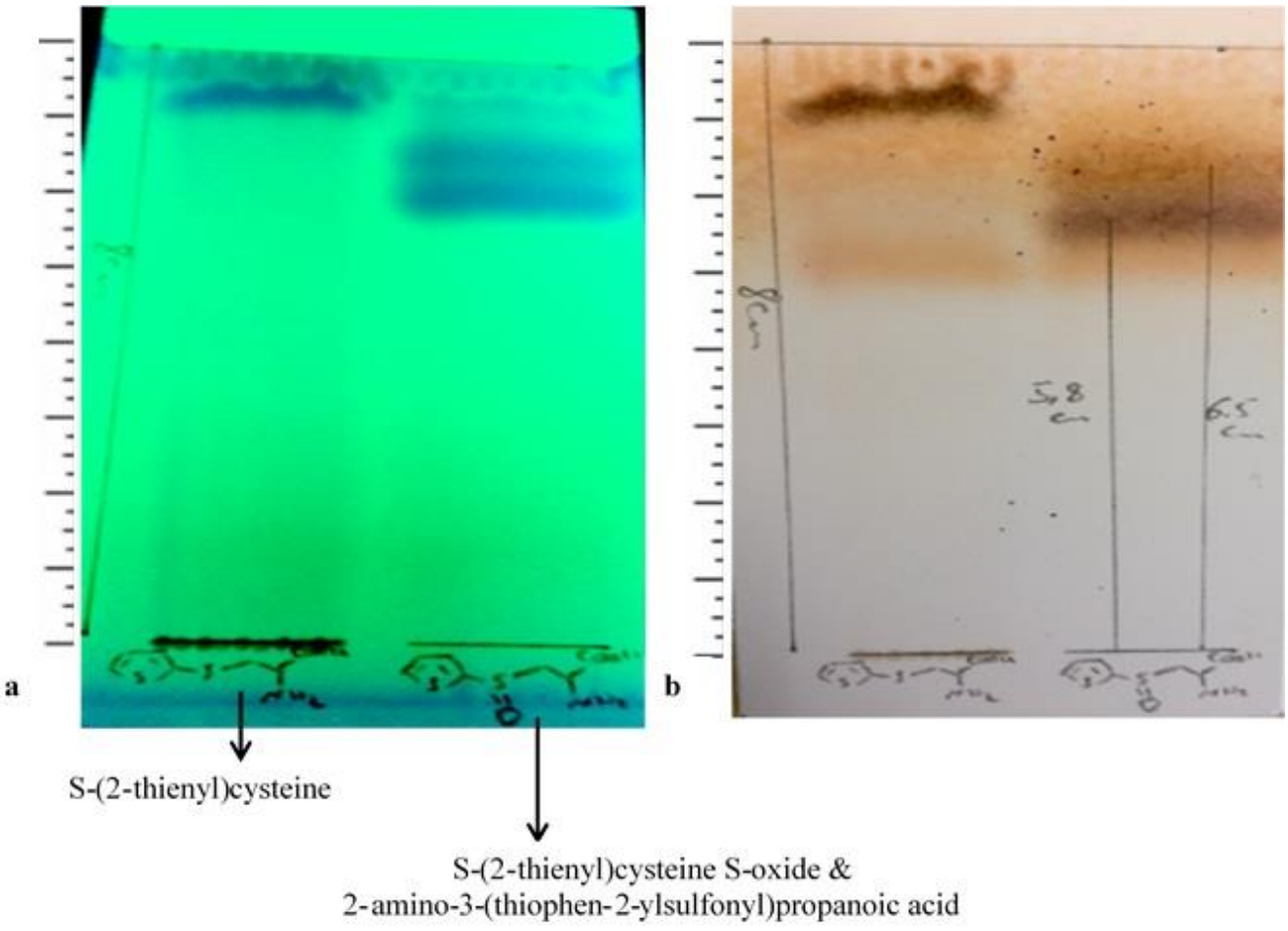


Figure 3.36 TLC analysis of S-(2-thienyl)cysteine-S-oxide. a) UV light detection (254 nm) and b) plate sprayed with ninhydrin.

3.7.2 ^{1}H-NMR analysis

The result of the TLC was further confirmed by the ^{1}H-NMR analysis. In the heteroaromatic part of the compound, protons H1′ δ = 7.41 ppm (md), H2′ δ = 7.23 ppm (md), and H3′ δ = 7.93 ppm (md), each with integral 1, were observed. Moreover, the signals H1–H3 belonging to the aliphatic part, each with integral 1, were detected: H1 at δ = 4.16 ppm (*J* = 2.52, 9.62 Hz, dd); the diastropic methylene hydrogens H2 and H3 at δ = 3.65 ppm (*J* = 2.52, 6.41 Hz, dd) and at δ = 3.91 ppm (*J* = 9.62, 5.72 Hz, dd), respectively (**Figure 3.37**).

3.7.3 ^{13}C-NMR analysis

When D_2O was used as a solvent, the mixture of sulfide, sulfoxide, and sulfonic compounds appeared in the ^{13}C-NMR spectrum (**Figure 3.38**). The chemical shift corresponding to carboxylic carbon was oberved at δ = 171.04 ppm. The carbons 4–7 belonging to the aromatic part of the thiophene group demonstrated strong chemical shifts at δ = 140.90 ppm, δ = 129.88 ppm, δ = 127.4 ppm, and δ = 132.9 ppm. The carbons of the methin and methylene groups showed the chemical shifts at δ = 50.72 ppm and δ = 56.88 ppm, respectively.

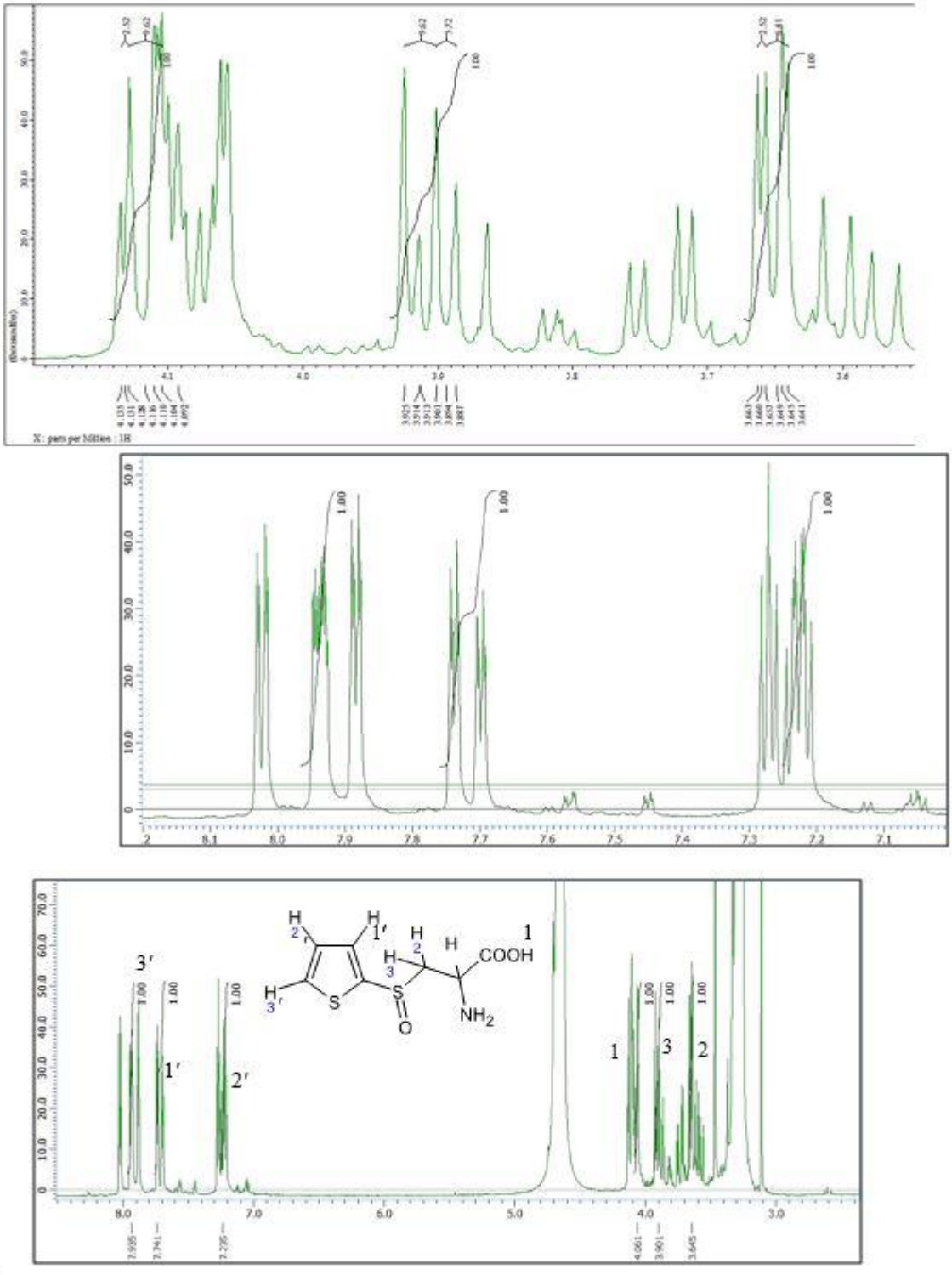


Figure 3.37 ^{1}H NMR spectra of S-(2-thienyl)cysteine-S-oxide.

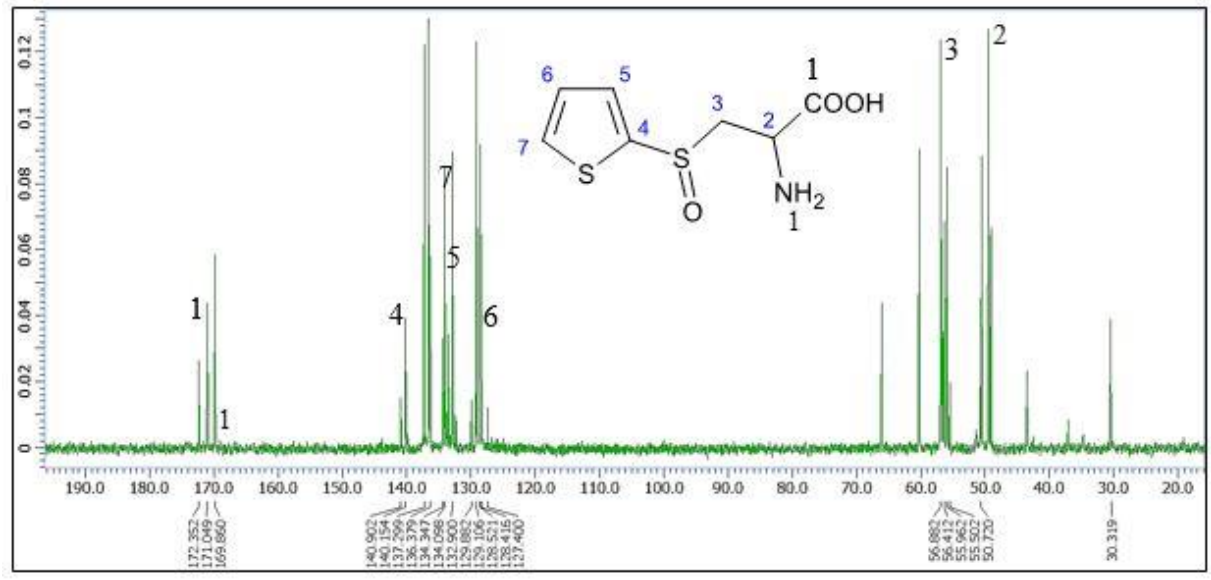


Figure 3.38 ^{13}C NMR spectra of S-(2-thienyl)cysteine-S-oxide.

3.7.4 IR analysis

The IR analysis revelaed stretching bands at 1138, 1496, 1626, 2900, 2992, 3088, 3250, and 3388 cm^{-1}. The band at 1138 cm^{-1} was related to the S=O group. The bands related to the C=C and C-H aromatic groups were visible at 1496 and 2992 cm^{-1}, respectively. The bands at 1626 and 3380 cm^{-1} corresponded to the C=O and NH_2 groups. The bands relating to the NH_2 group were observed at 3088 and 3250 cm^{-1}, respectively. The weak band at 2900 cm^{-1} belonged to the C-H alkane group (**Figure 3.39**).

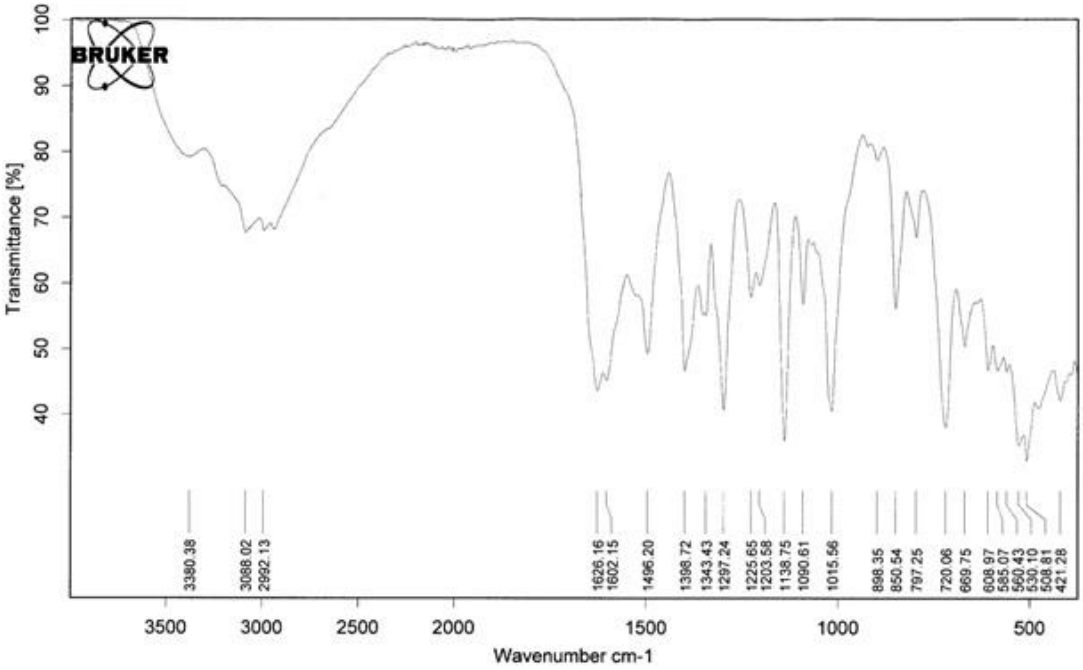


Figure 3.39 IR spectrum of S-(2-thienyl)cysteine-S-oxide.

3.7.5 ESI-MS analysis

During the ESI-MS analysis, the mass spectrum displayed the positive ion peak of the product S-(2-thienyl)cysteine-S-oxide at *m/z* 220 $[M+H]^+$, *m/z* 242 $[M+Na]^+$, and *m/z* 258 $[M+K]^+$ (**Figure 3.40**).

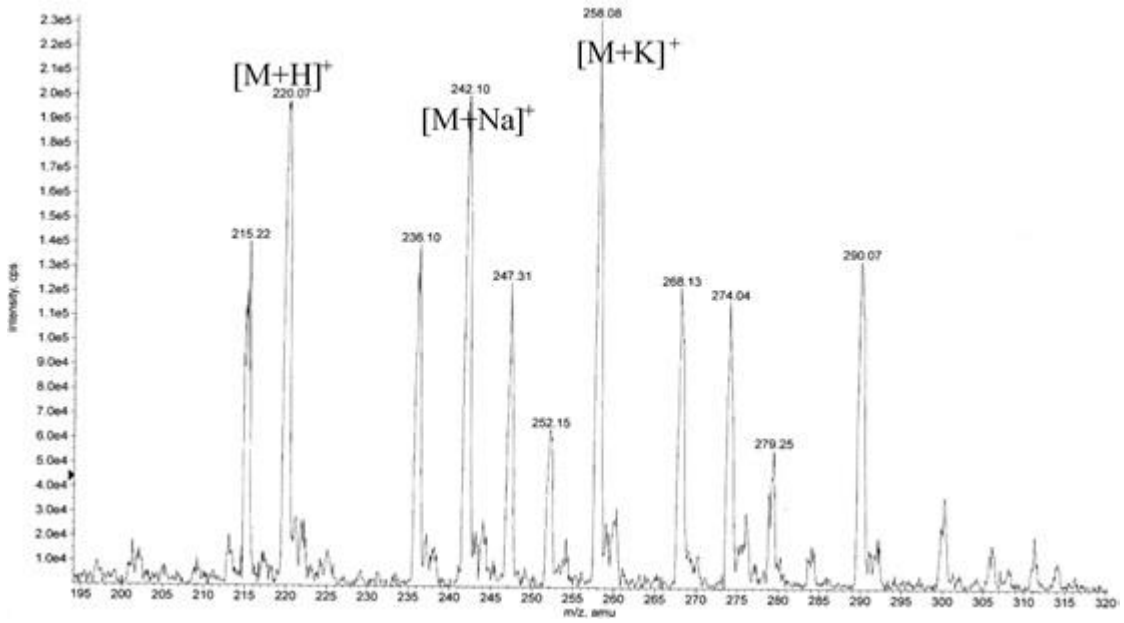
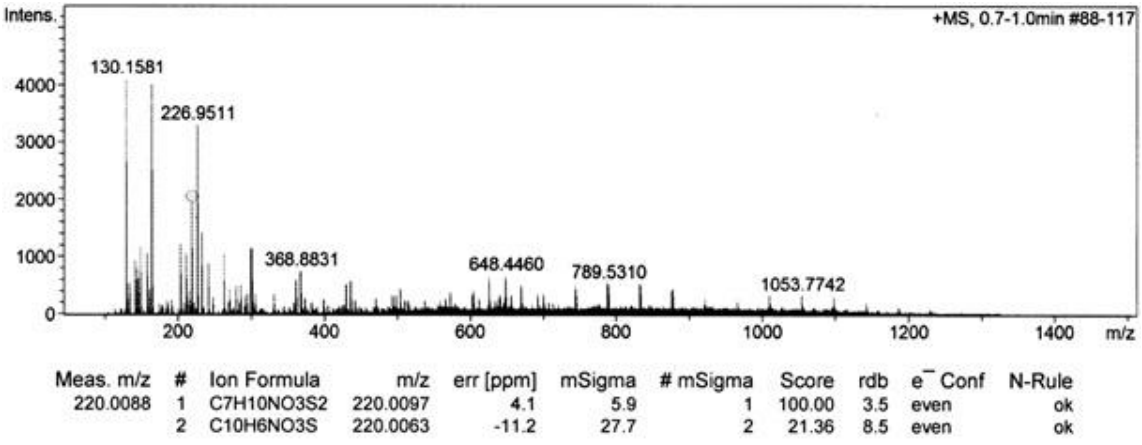


Figure 3.40 MS analysis of S-(2-thienyl)cysteine-S-oxide.

3.7.6 HR-MS analysis

According to the results of the HR-MS analysis, the ratio of molecular weight to molecular charge (*m/z)* correlated with the molecular formula of $C_7H_{10}NO_3S_2$. Its proposed value was equal to *m/z* 220.0097 and the measured value was *m/z* 220.0088 with an acceptable difference of about 0.9 mDa (**Figure 3.41**).



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
220.0088	1	C7H10NO3S2	220.0097	4.1	5.9	1	100.00	3.5	even	ok
	2	C10H6NO3S	220.0063	-11.2	27.7	2	21.36	8.5	even	ok

Figure 3.41 HR-MS analysis of S-(2-thienyl)cysteine-S-oxide.

3.8 1-(Triisopropylsilyl)pyrrole

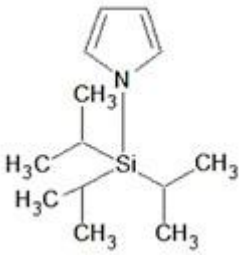


Figure 3.42. 1-(Triisopropylsilyl)pyrrole

3.8.1 ^{1}H-NMR analysis

The H-NMR analysis was conducted using $CDCl_3$ (chloroform D) as the solvent. The chemical shifts indicating a pyrrole ring, each with integral 2, were characterized by the signals at δ = 6.57 ppm and δ = 6.7.04 ppm. In addition, hydrogens 3 of the isopropyl group were identified with integral 18 at δ = 1.38 ppm and hydrogen 3 of the methyl group with integral 3 at δ = 1.72 ppm (**Appendix 16**).

3.8.2 ESI-MS analysis

As shown in **Appendix 17**, the basic peak in ESI-MS analysis was observed at *m/z* 224.25 $[M+H]^+$ corresponding to the desired product 1-(triisopropylsilyl)pyrrole.

3.9 Hydrogenation

3.9.1 Hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

3.9.1.1 Hydrogenation with pd/C and hydrogen

3.9.1.1.1 ***ESI-MS analysis***

The ESI-MS analysis conducted in a mixture of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide and (2-amino-3-[(1-benzyl-1*H*-pyrrol-2yl)sulfonyl]propanoic acid) did not result in the expected product at *m/z* 203 $[M+H]^+$. However, the results showed two peaks: a peak at *m/z* 277 $[M+H]^+$ correlated to S-(N-benzylpyrrol-2-yl)cysteine-S-oxide, while the other one. at *m/z* 309 $[M+H]^+$, indicated the occurrence of 2-amino-3-[(1-benzyl-1*H*-pyrrol-2yl)sulfonyl]propanoic acid (**Appendix 18**).

3.9.1.2 Hydrogenation of N-benzylpyrrole

3.9.1.2.1 ^{1}H-NMR analysis

The ^{1}H-NMR analysis was performed with $CDCl_3$ as a solvent. The ^{1}H-NMR spectrum displayed the signals of a pyrrole ring with integral 2 at δ = 6.17 and δ = 6.67 ppm. Additionally, the aromatic toluene ring was characterized by the signals at δ = 7.1 and δ = 7.3 ppm, each with integral 2. The signals present at δ = 2.14 ppm with integral 3 corresponded to the methyl hydrogen group of the toluene ring, which indicated the successful removal of the benzyl group from the N-benzylpyrrole compound (**Appendix 57**) (**Figure 3.43**).

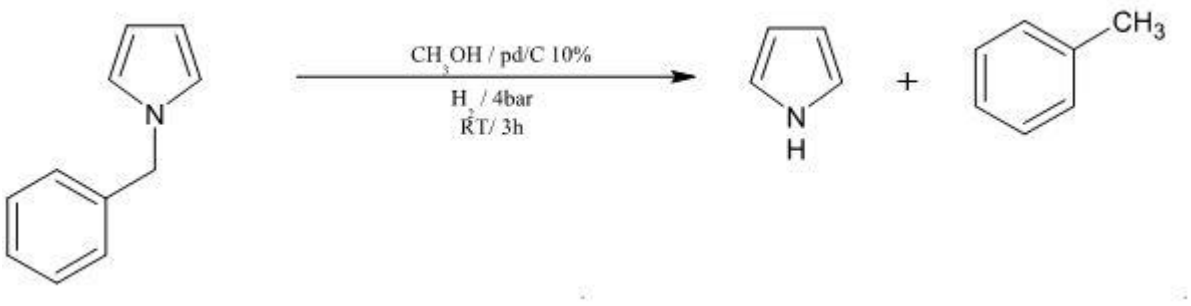


Figure 3.43. Hydrogenation of N-benzylpyrrole

Table 3.3 ESI-MS and TLC analyses of further synthesized products. The peaks observed following the analysis indicated as * in the table.

Compound	Molecular weight (g/mol)	Expercted peak (*m/z*)					TLC	Appendix Nr.
		Q+ (m/z)				Q- (*m/z*)		
		$[M+H]^+$	$[2M+H]^+$	$[M+Na]^+$	$[M+K]^+$			
S-(N-Bocpyrrol-2-yl)cysteine	284	285		307	323			20
S-(3-furanyl)cysteine								
1-H-pyrrole-sulfonyl chloride								
Furan-3-sulfonyl chloride								
S-(2-furanyl)cysteine								
Cbz-serine benzylester-O-trifilate	461	462		484				21
Lithium furan-2-thiolate	106	107		129				22
2-[(tert-butoxycarbonyl)amino]-3-(1-H-pyrrole-2-ylsulfanyl)propanoic acid							Not applicated	
S-(2-pyrrolyl)cysteine	186	187		209		185		23/24
S-(N-methylpyrrol-2-yl)cysteine							Not applicated	
Trifluoromethylsulfonyl-serine with triethylamine	237	238		260	276			25
O-trifluoromethylsulfonyl-serine with 2,6-lutidine							Not applicated	
S-(3-thienyl)cysteine	203	204		226	242			26
Boc-S-(N-methylpyrrol-2-yl)cysteine	300	301		323	339	299		27/28
Bromo-N-methylpyrrole	160	161*	321*		199*			29
N- (tert-butoxycarbonyl)-O-trifluoromethylsulfonyl-serine with 2,6-lutidine	237	238		260	276			30
S-(3-furanyl)cysteine								
S-(N-triisopropylsilypyrrol-2-yl)cysteine							Not applicated	
S-(2-thienyl)cysteine								
S-(2-imidazolyl)cysteine	187	188		210		186		31/32

Compound	Molecular weight (g/mol)	Expercted peak (*m/z*)					TLC	Appendix Nr.
		Q+ (m/z)				Q- (*m/z*)		
		$[M+H]^+$	$[2M+H]^+$	$[M+Na]^+$	$[M+K]^+$			
S-(N-methylimidazol-2-yl)cysteine	201	202		224		200		33/34
S-(2-thiazolinyl)cysteine	206	207		229				35
S-(3-thienyl)cysteine							Not applicated	
Oxidation								
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with H_2O_2 in acetic acid)	292	293*		315*	331*			36
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with $FeCl_3$ and H_5IO_6)	292	293		315				37
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with MMPP)	292	293*						38
S-(N-methylpyrrol-2-yl)cysteine (oxidation with H_2O_2 in water)	216	217						39
S-(N-methylpyrrol-2-yl)cysteine (oxidation with H_2O_2 in acetic acid)	216	217						40/41
S-(N-methylpyrrol-2-yl)cysteine (oxidation with cumene hydroperoxide in H_2O)	216	217						42
S-(N-methylpyrrol-2-yl)cysteine (oxidation with MMPP)	216	217						43
S-(N-methylpyrrol-2-yl)cysteine (oxidation with TAPC in H_2O_2)	216	217						44
Hydrogenation								
Catalytic transfer hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide	202	203	405	225	241			52

Table 3.4 ^{1}H NMR and TLC analyses of further synthesized products.

Compound	Solvent	Chemical shift (ppm)		TLC	Appendix nr.
		Aromatic (δ 6–8)	Aliphatic (δ 1–4)		
S-(N-Bocpyrrol-2-yl)cysteine	D2O	unobserved	observed		45
S-(3-furanyl)cysteine	D2O	unobserved	observed		46
1-H-pyrrole-sulfonyl chloride	D2O	unobserved	observed		47
Furan-3-sulfonyl chloride	D6-Acetone	unobserved	observed		48
S-(2-furanyl)cysteine	D2O	unobserved	observed		49
Cbz-serine benzylester-O-trifilate		unobserved	observed		
Lithium furan-2-thiolate					
2-[(tert-butoxycarbonyl)amino]-3-(1-H-pyrrole-2-ylsulfanyl)propanoic acid				Not applicated	
S-(2-pyrrolyl)cysteine					
S-(N-methylpyrrol-2-yl)cysteine				Not applicated	
Trifluoromethylsulfonyl-serine with triethylamine					
O-trifluoromethylsulfonyl-serine with 2,6-lutidine				Not applicated	
S-(3-thienyl)cysteine	D2O		Observed		50
Boc-S-(N-methylpyrrol-2-yl)cysteine					
Bromo-N-methylpyrrole					
N- (tert-butoxycarbonyl)-O-trifluoromethylsulfonyl-serine with 2,6-lutidine					
S-(3-furanyl)cysteine	D2O	unobserved	Observed		51
S-(N-triisopropylsilypyrrol-2-yl)cysteine				Not applicated	
S-(2-thienyl)cysteine					
S-(2-imidazolyl)cysteine					
S-(N-methylimidazol-2-yl)cysteine					
S-(2-thiazolinyl)cysteine					

Compound	Solvent	Chemical shift (ppm)		TLC	Appendix nr.
		Aromatic (δ 6–8)	Aliphatic (δ 1–4)		
S-(3-thienyl)cysteine				Not applicated	
Oxidation					
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with H2O2 in acetic acid)					
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with FeCl3 and H5IO6)					
S-(N-benzylpyrrol-2-yl)cysteine (oxidation with MMPP)					
S-(N-methylpyrrol-2-yl)cysteine (oxidation with H2O2 in water)					
S-(N-methylpyrrol-2-yl)cysteine (oxidation with H2O2 in acetic acid)					
S-(N-methylpyrrol-2-yl)cysteine (oxidation with cumene hydroperoxide in H2O)					
S-(N-methylpyrrol-2-yl)cysteine (oxidation with MMPP)					
S-(N-methylpyrrol-2-yl)cysteine (oxidation with TAPC in H2O2)					
Hydrogenation					
Catalytic transfer hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide					

3.9.2 Catalytic transfer hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

3.9.2.1 MS analysis

The peaks representing 2-amino-3-[(1-benzyl-1*H*-pyrrol-2yl)sulfonyl]propanoic acids, which indicate the primary compound of S-(N-benzylpyrrol-2-yl)cysteine, were observed at *m/z* 309 $[M+H]^+$ and at *m/z* 277 $[M+H]^+$, respectively. As illustrated in the MS spectrum, the expected peak associated with S-(N-benzylpyrrol-2-yl)cysteine-S-oxide at *m/z* 203 $[M+H]^+$ was not detected (**Appendix 52**).

Tables 3.3 and **3.4** illustrate unsuccessful attempts to synthesize some heteroaromatic cysteine and cysteine sulfoxides.

3.10 Enzymatic digestion

All of the synthesized products described in the above sections were tested by alliinase from *A. sativum*.

3.10.1 Enzymatic digestion of S-(2-thienyl)cysteine-S-oxide

According to the results of the HPLC-MS analysis (see **Appendixes 53–61**), the results of three different HPLC-MS analyses, conducted after 2, 4, and 6 hours, respectively, demonstrated that the optimal test was performed within a time period of 2 h.

The enzymatic digestion was performed including the reaction test, and the positive and negative control.

The reaction test consists of S-(2-thienyl)cysteine-S-oxide plus alliinase in the alliinase buffer, the positive control consists of alliin plus alliinase in the alliinase buffer, and the negative control consists of alliinase in the allinase buffer.

Unexpectedly, in the negative control, peaks emerged at 11.10 min.(+), 27 min (+), 28.6 min (+), and 21.6 min (++++). The most significant peak formed at 21.6 min (++++), which indicated a compound with the molecular weight of 135 g/mol (**Table 3.5a** and **Appendix 62**). In the positive control (**Appendix 63**), the compounds allicin at *m/z* 163 $[M+H]^+$ (**Table 3.5b**) and ajoene at *m/z* 235 $[M+H]^+$ (**Table 3.5c**) were detected at 21.8 min. (++++) (**Appendix 64**) and 25.8 min. (+++) (**Appendix 65**), respectively; this indicated a compound with the molecular weight of 135 g/mol (**Table 3.5a** and **Appendix 66**). In order to check the possible ionization

of alliicin, the (-Q)-trap analysis was conducted and the results demonstrated a less intensive signal correlated to the molecular weight of 161 (**Appendix 67**). The findings also confirmed that the compound with the molecular weight of 135 g/mol had to be considered in all of the calculations. The purity of S-(2-thienyl)cysteine-S-oxide was calculated to be 47.3% (**Appendix 15**). After the reaction of S-(2-thienyl)cysteine-S-oxide with alliinase extracted from *A. sativum*, considering the compound with the molecular weight of 135 g/mol, the only detected compounds were S-thiophen-2-yl thiophene-2-sulfinothioate at *m/z* 247 $[M+H]^+$) (**Table 3.5d**) and 2,2´-(1,2,-dioxido-1λ^4, 2λ^4-disulfane-1,2-diyl)dithiophene at *m/z* 263 $[M+H]^+$ (**Table 3.5e**), as expected. The peak that formed at 27 min. (+++) corresponded to the compound linked to the sulfoxide group (**Appendix 68**). The second remarkable peak appeared at 26 min. (++) and matched the compound related to the disulfide-S-S´-dioxide group (**Appendix 69**), which is an oxidation product after alliinase reaction.

3.10.2 Enzymatic digestion of other synthesized products

Enzymatic digestion was performed on S-(2-pyrrolyl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine-S-oxide, and S-(2-thienyl)cysteine.

3.10.2.1 Enzymatic digestion of S-(2-pyrrolyl)cysteine

Appendix 70 contains the results of the HPLC-MS analysis of the enzymatic digestion of S-(2-pyrrolyl)cysteine. None of the expected products corresponded to disulfide-S-oxide (at *m/z* 213 $[M+H]^+$), S-dipyrrolyldisulfide (at *m/z* 197 $[M+H]^+$, or at *m/z* 313 $[M+135+H]^+$) (**Appendix 71**). However, the major peaks were detected at 17 min (++++) (at *m/z* 222 $[M+H]^+$) (**Table 3.5f** and **Appendix 72**), 18 min (++) (at *m/z* 231 $[M+H]^+$) (**Table 3.5g** and **Appendix 73**), and at 21 min (+) (at *m/z* 231 $[M+H]^+$) (**Table 3.5g** and **Appendix 74**), with fragments different than those of the previous peaks at 26 min (at *m/z* 226 $[M+H]^+$) (**Table 3.5h** and **Appendix 75**) and at 32 min (++) (at *m/z* 294 $[M+H]^+$) (**Appendix 76**).

3.10.2.2 Enzymatic digestion of S-(N-methylpyrrol-2-yl)cysteine

The chromatogram obtained from the HPLC-MS analysis associated with the enzymatic digestion of S-(N-methylpyrrol-2-yl)cysteineis is shown in **Appendix 77**. None of the expected products corresponding to disulfide-S-oxide (at *m/z* 241 $[M+H]^+$), S-disulfide (at *m/z* 225 $[M+H]^+$, or even at *m/z* 360 $[M+135+H]^+$) were detected (**Appendix 78**). Nevertheless,

significant peaks related to unexplained products were observed at 23.72 min (+++) (at *m/z* 231 $[M+H]^+$) (**Table 3.5i** and **Appendix 79**), 24.4 min (++++) (at *m/z* 222 $[M+H]^+$) (**Table 3.5j** and **Appendix 80**), 27.5 min (+) (at *m/z* 266 $[M+H]^+$) (**Table 3.5k** and **Appendix 81**), and 33 min (+++) (at *m/z* 294 $[M+H]^+$) (**Table 3.5l** and **Appendix 82**).

3.10.2.3 Enzymatic digestion test of S-(N-benzylpyrrol-2-yl)cysteine

Appendix 83 illustrates the chromatogram obtained through the HPLC-MS analysis relevant to the enzymatic digestion test of S-(N-benzylpyrrol-2-yl)cysteine. The evidence indicated that no expected products corresponded to disulfide-S-oxide (at *m/z* 393 $[M+H]^+$), disulfide (at *m/z* 377 $[M+H]^+$ and/or even at *m/z* 512 $[M+135+H]^+$) (**Table 3.5m** and **Appendix 84**). However, two intensive peaks belonging to unidentified products—both observable in previous compounds—were detected at 27 min(+) (at *m/z* 266 $[M+H]^+$) (**Appendix 85**) and 33 min (++++) (at *m/z* 294 $[M+H]^+$) (**Table 3.5n** and **Appendix 86**).

3.10.2.4 Enzymatic digestion test of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide

Unlike in the previous reaction, the alliinase reacted with S-(N-benzylpyrrol-2-yl)cysteine-S-oxide as a cysteine sulfoxide (**Appendix 87**) and none of the expected products corresponding to disulfide-S-oxide (at *m/z* 393 $[M+H]^+$), disulfide (at *m/z* 377 $[M+H]^+$ or at *m/z* 528 $[M+135+H]^+$) were observed (**Appendix 88**). However, two major peaks corresponding to unidentified products and observable in the compound with the pyrrole ring were found at 20.9 min (++++) (at *m/z* 231 $[M+H]^+$) (**Table 3.5o** and **Appendix 89**) and 33.3 min (+++) (at *m/z* 294 $[M+H]^+$) (**Table 3.5p** and **Appendix 90**).

3.10.2.5 Enzymatic digestion test of S-(2-thienyl)cysteine

Appendix 91 illustrates the results of the HPLC-MS analysis connected to the enzymatic digestion test of S-(2-thienyl)cysteine. Although none of the expected products corresponding to disulfide-S-oxide (at *m/z* 247 $[M+H]^+$), S-dithienylsulfide (at *m/z* 231 $[M+H]^+$ or even at *m/z* 366 $[M+135+H]^+$) (**Appendix 92**) were observed, the significant peaks related to unidentified products were detected at 6.2 min (at *m/z* 155 $[M+H]^+$) (**Table 3.5q** and **Appendix 93**), at 39.6 min (at *m/z* 273 $[M+H]^+$) (**Table 3.5r** and **Appendix 94**), and at 54.38 min (at *m/z* 299 $[M+H]^+$) (**Table 3.5s** and **Appendix 95**).

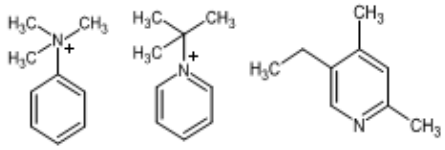
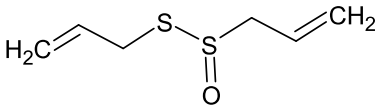
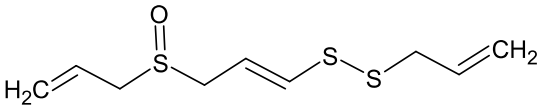
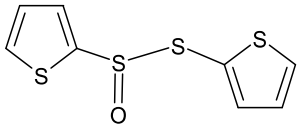
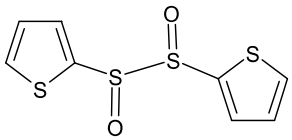
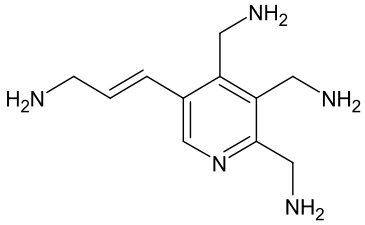
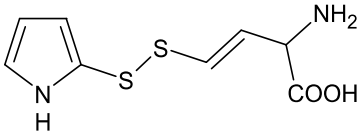
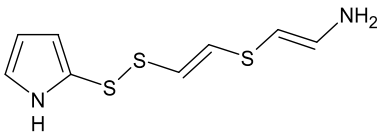
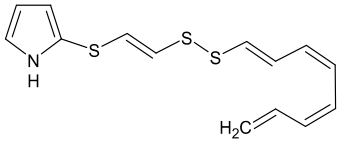
3.10.2.6 HR-MS analysis of compound at *m/z* 136 $[M+H]^+$

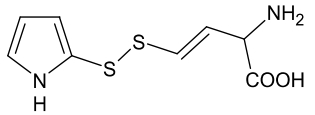
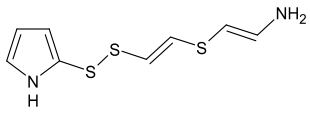
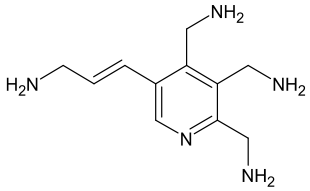
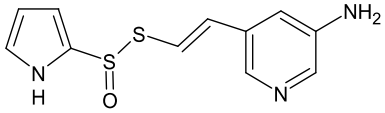
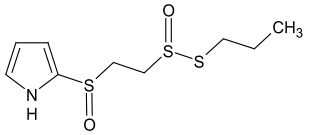
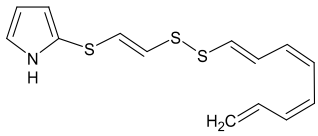
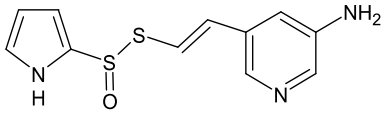
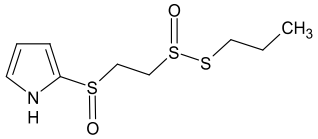
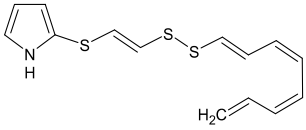
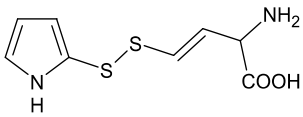
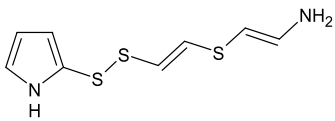
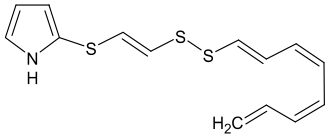
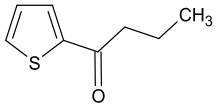
To identify the compound with the molecular weight of 135 g/mol, HR-MS analysis was conducted (**Appendix 96**): it showed a *m/z* ratio of 136.1128 $[M+H]^+$, which indicates the existence of $C_9H_{14}N$ with a theoretical value of *m/z* 136.1121 and a negligible difference of 0.7 mDa (**Table 3.5a**).

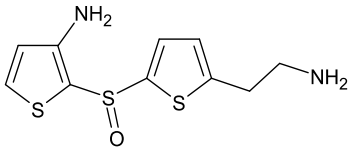
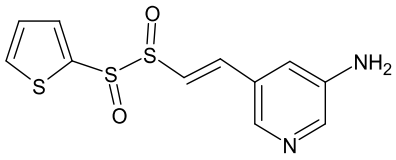
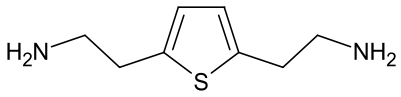
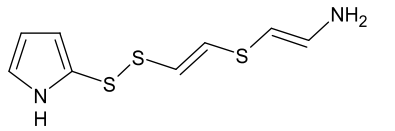
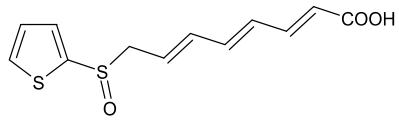
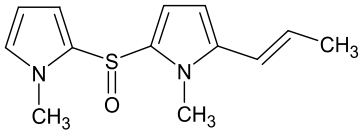
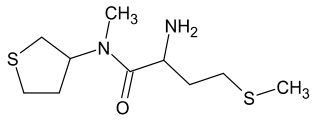
3.10.2.7 HR-MS analysis of compound at *m/z* 171 $[M+H]^+$

HR-MS analysis was carried out to detect the compound with the molecular weight of 170 g/mol (**Appendix 97**). The results of the HR-MS analysis showed that a *m/z* ratio of 171,0989 $[M+H]^+$ represented four different compounds, two of which contained fluoride. One did not contain any nitrogen and sulfur, and the other one with the molecular formula of $C_8H_{15}N_2S$ seemed to be the desired substance (**Table 3.5t**).

Table 3.5 Results of enzymatic digestionusing HPLC-MS on synthesized products. Alliinase was extracted from *A. sativum* for all analyses (Krest and Keusgen, 1999b).

Substrate	(*m/z*) $[M+H]^+$	Retention Time (min.)	Peak Intensity	Intendet Molecular Structure
a) Alliinase (negative control)	**a)** 136	21.6	(++++)	
b) Alliin (positive control)	**b)** 163	21.8	(++++)	
	c) 235	25.8	(+++)	
S-(2-thienyl)cysteine-S-oxide	**d)** 247	27	(+++)	
	e) 263	26	(++)	
S-(2-pyrrolyl)cysteine	**f)** 222	17	(++++)	
	g) 231	18	(++)	
		21	(+)	
	h) 294	32	(++)	

S-(N-methylpyrrol-2-yl)cysteine	**i)** 231	23.72	(+++)	 
	j) 222	24.4	(++++)	
	k) 266	27.5	(+)	 
	l) 294	33	(+++)	
S-(N-benzylpyrrol-2-yl)cysteine	**m)** 266	27	(+)	 
	n) 294	33	(++++)	
S-(N-benzylpyrrol-2-yl)cysteine S-oxide	**o)** 231	20.9	(++++)	 
	p) 294	33.3	(+++)	
S-(2-thienyl)cysteine	**q)** 155	6.2	(++++)	

r) 273	39.6	(+)	
s) 299	54.3	(-)	
t) 171	____	(-)	
u) 269	____	(-)	 
v) 249	____	(-)	 

3.10.2.8 HR-MS analysis of compound at *m/z* 269 $[M+H]^+$

In order to identify the compound with the molecular weight of 268 g/mol (**Appendix 98**) the HR-MS analysis was carried out: it showed that a *m/z* of 269.0274 [M+H]+ is possible for 33 different compounds. Moreover, the compounds containing nitrogen and sulfur with the molecular formulas of $C_{12}H_{13}O_3S_2$, and $C_{11}H_{13}N_2S_3$ are the most desirable compounds (**Table 3.5u).**

3.10.2.9 HR-MS analysis of compound at *m/z* 249 $[M+H]^+$

In order to identify the compound with the molecular weight of 248 g/mol, the HR-MS analysis was conducted (**Appendix 99**): it revealed that *m/z* of 249.1094 $[M+H]^+$ correlates with 7 different compounds. Moreover, the compounds with the molecular formulas of $C_{13}H_{17}N_2OS$ and $C_{10}H_{21}N_2O_2S_2$ are the most significant ones. These compounds have theoretical values of *m/z* 249.1056 and *m/z* 249.1090 with differences in amounts of about 3.8 mDa and 0.4 mDa, respectively. (**Table 3.5v**).

3.10.2.10 HR-MS analysis of compound at *m/z* 222 $[M+H]^+$

The HR-MS analysis was conducted to identify the compound with the molecular weight of 221 g/mol (**Appendix 100**): it revealed that a *m/z* ratio of 222.1711 $[M+H]^+$ possibly correlates with three different compounds. The most plausible compound is defined by the molecular formula of $C_{11}H_{20}N_5$ with a theoretical value of *m/z* 222.1713 and a difference of 0.2 mDa (**Table 3.5j**).

4. Discussion

4.1 Synthesis of heteroaromatic cysteine sulfoxides

The synthesis of different types of five-ring heteroaromatic cysteine sulfoxides is rather interesting, as they are significant and naturally available in some wild species of *Allium*, such as *A. giganteum* (Kubec et al., 2011 and *A. rosenorum* (Jedelská et al., 2008), and also very likely available in *A. carolinanum* and *A. macleanii* (Vogt, 2008-doctoral thesis). The synthesis of any of the aromatic homologous compounds was also the aim of this study. These pyrrole compounds have been reported to have antidiabetic (Holland, 1981) and antibiotic activities, particularly in *A. giganteum* against *Streptococcus sp.* (Kubec et al., 2011).

The pyrrole cysteine sulfoxides, such as S-(3-pyrrolyl)cysteine-S-oxide isolated from *A. rosenorum* subgen. *Melanocrommyum* by Jedelská et al. (2008), were considered as suitable substrates for alliinase. They affect the formation of the red pigment, the structure of which has to date not been fully understood.

The main goal of this research was to synthesize S-(2-pyrrolyl)cysteine-S-oxide or S-(3-pyrrolyl)cysteine-S-oxide and their homologs in order to understand the mechanisms as well as the structure of the red pigment under alliinase reaction (Jedelská et al., 2008). To synthesize these compounds, the TLC, ESI-MS, HR-MS, ^{1}HNMR ^{13}CNMR, IR, HPLC, and HPLC-MS analyses were implemented. Pyrrole, methyl pyrrole, benzyl pyrrole, and thiophene-2-thiol were selected as primary educts.

Higher rate of reactivity, quick polymerization, oxidation, and light instability make the synthesis of pyrrole compounds difficult (Dennstedt and Voigtländer, 1984). Therefore, it is essential to find a synthesis method that can eliminate these complications. The method described here for the synthesis of four pyrrole compounds, including S-(2-pyrrolyl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine, and S-(N-benzylpyrrol-2-yl)cysteine S-oxide (after Rudyakova et al., 2008) is considered essential because of the application of non-protected amino acids. Of all the products, S-(2-thienyl)cysteine demonstrated the highest yield of 93%, and S-(N-methylpyrrol-2-yl)cysteine had the lowest yield of 22%.

The oxidation approach failed to synthesize the compound S-(2-pyrrolyl)cysteine-S-oxide from the primary educt (S-(2-pyrrolyl)cysteine) because of its high instability. Five other homologous products, including S-(N-methylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine-S-oxide, S-(2-thienyl)cysteine, and S-(2-

thienyl)cysteine S-oxide were successfully synthesized. The effect of alliinase from *A. sativum* was tested on the abovementioned compounds.

4.2 3-Chloro-L-alanine as the basic reagent for the synthesis

In the present study, 3-chloro-L-alanine (Figure 4.1a) was considered a fundamental product for the synthesis of other products. The existence of chloride, as a suitable leaving group in the nucleophilic substitution reactions, is one of the main advantages of 3-chloro-L-alanine. L-serine (**Figure** 4.1b), saturated with HCl (for more details see methods), was used as a primary educt. Despite the high structural similarity of 3-chloro-L-alanine and L-serine (the only difference is the presence of the Cl and OH groups, respectively), the hydrogens of methin and methylene groups demonstrated high variability in the chemical shifts in the ^{1}HNMR analysis. This interesting phenomenon is discussed further in the continuation of this section.

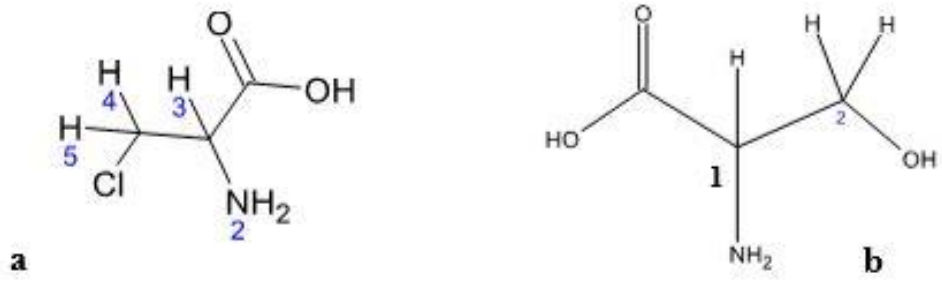


Figure 4.1 Molecular structure of 3-chloro-L-alanine (a) and L-serine(b)

The ^{1}H-NMR spectra of 3-chloro-L-alanine and L-serine are presented in **Figure** 4.2. L-serine showed one signal belonging to the hydrogen of the methin group with integral 1 (δ = 3.75 ppm, md) and a peak of the methylene hydrogens with integral 2 (δ = 3.9 ppm, md). Moreover, 3-chloro-L-alanine displayed remarkable differences in chemical shifts of the diastereotic methylene hydrogens [integral 1 (H4, δ = 4 ppm, *J* = 3.43, 12.82 Hz, dd) and (H5, δ = 4.11 ppm, *J* = 4.35, 12.82 Hz, dd)] and the methin hydrogen [integral 1 (H3, δ = 4.45 ppm, *J* = 3.43, 4.35 Hz, dd)] (**Figure 3.2**). In compression, two significant differences were observed: i) the exchange of the chemical shifts between methin and methylene hydrogens and; ii) the diastereotopic methylene hydrogens in 3-chloro-L-alanine, which was represented by two signals (dd).

The difference in multiplicity of peaks could be due to the following reasons: i) D_2O as a solvent for both substances may have caused the replacement of the hydroxyl group (OH) proton with

that of deuterium. Using DMSO instead of D_2O as a solvent could help optimize this phenomenon; ii) the coupling constants of the peaks show that they can be formed very closely and result in a multi doublet.

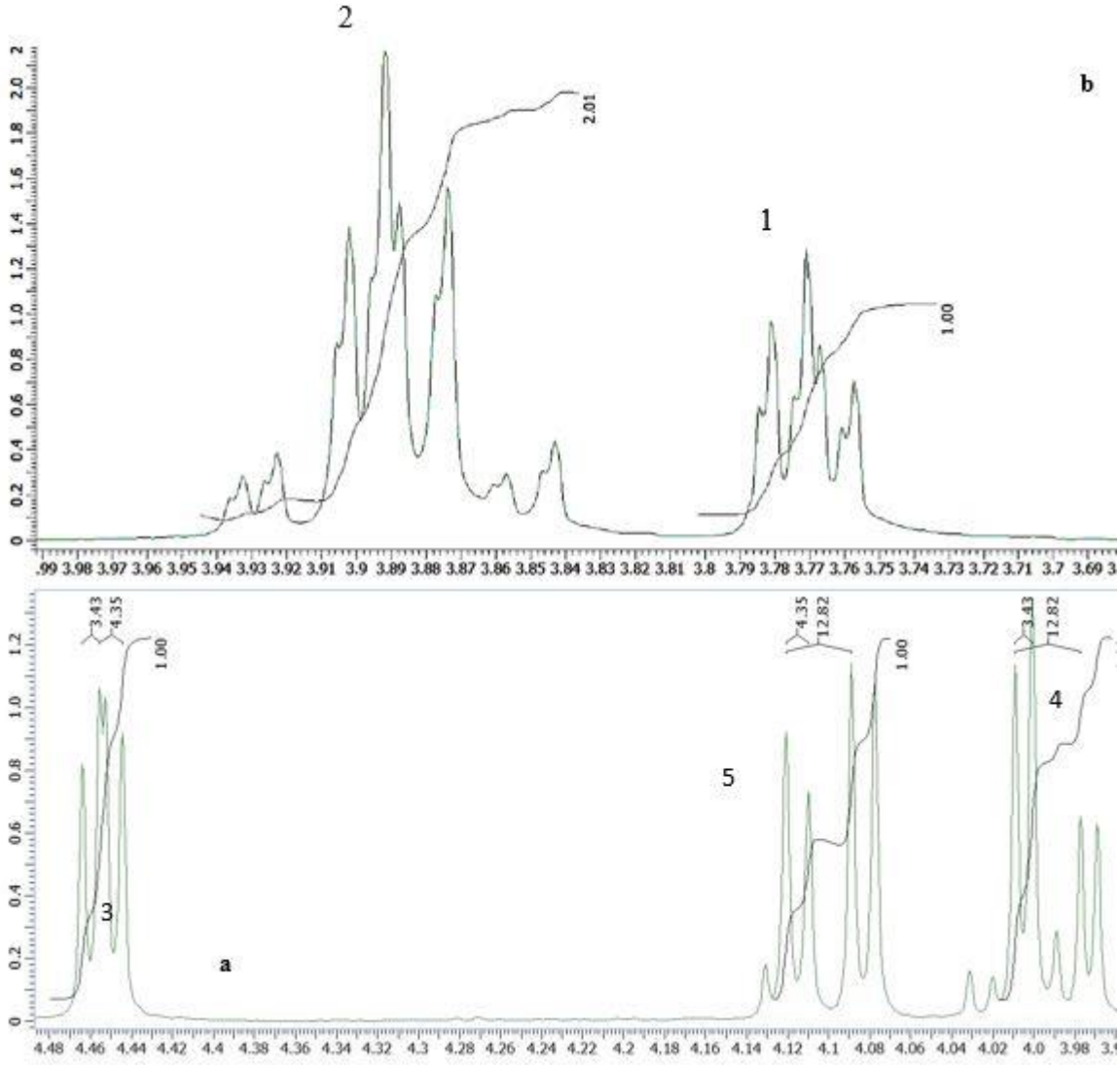


Figure 4.2 ^{1}H-NMR spectra of 3-chloro-L-alanine (a) and L-serine (b).

Using EX500 instead of EX400 would optimize the visualization of this phenomenon to some extent. Current theories do not seem capable of explaining why chemical shifts of protons in L-serine and 3-chloro-L-alanine differ significantly. However, the saturation of 3-chloro-L-alanine with HCl may represent the dipolar ion in the reaction, in which the amine and the carboxyl appear as $–NH_3^+$ and –COOH, respectively. The difference in the chemical shift of these two compounds can be explained to some extent by the difference in the electronegativity of the chloride and hydroxyl groups. The electronegativity of the chloride group is constant,

while that of the hydroxyl group is relative and differentiable for each compound. In general, the weaker the H-bond appeared, the higher the electronegativity of the OH group was observed (Krygowski and Szatyłowicz, 2006). And the higher the electronegativity of the OH group in L-serine was observed, the higher the number of adjacent diastereotopic hydrogens that were shifted to downfield. Despite the theories mentioned above, it is not clear why the chemical shift of the hydrogens of methylene and methin should be different in two almost identical compounds (L-serine and 3-chloro-L-alanine).

4.3 Reaction mechanisms

To date, no acceptable mechanism has been presented for the synthesis of the three main products: S-(2-pyrrolyl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, and S-(N-benzylpyrrol-2-yl)cysteine. Here, it is hypothesized that the reaction mechanism may take place in following steps: First, during an intermediate reaction, the iodide in the mixture of KI/I_2 acts as an oxidative and convert thiourea to sulfide dimer. As previously demonstrated, thiourea can be converted to sulfide dimer by using a halogen (Soroka & Goldeman, 2005; Hunter & Jones, 1930). If this scenario explains the formation of sulfide dimer from KI/I_2 and thiourea, the second step occurs with the reaction of disulfide and aromatic pyrrole ring in position 2, resulting in an electrophilic aromatic substitution (SnEAr) reaction. Also, the pseudothiourea is considered an intermediate product the reaction and should last for three hours under argon atmosphere at 30–40˚C. The temperature control throughout the reaction is critical. Since the pyrrole ring is highly reactive, the reaction atmosphere should be kept constant and alkalinized by adding hydrazine and NaOH. Hydrazine is necessary for conducting the reaction, as it attaches to the disulfide bonds in pseudothiourea and forms a tetraammine compound. By splitting the sulfur atom from the thiolate, the electron pair shifts from the amine group to sulfur. Lastly, 3-chloro-L-alanine is added to the mixture and heated for two hours in a highly alkaline environment. This condition is suitable for a simple nucleophilic substitution II reaction (SN_2), in which the chloride acts as the leaving group and thiolate acts as the nucleophilic group. Afterwards, the thiolate is replaced by chloride, forming the desired product (**Figure** 4.3).

The other possible but unlikely mechanism for this reaction might be as follows: the reaction is assumed to start with I_2 binding to the pyrrole ring in position 2. Next, thiourea is replaced by I_2 with the use of sulfide dimer due to a nucleophilic substitution reaction in aromatic compounds. However, this scenario does not seem feasible due to the lack of substituents with –M-effect.

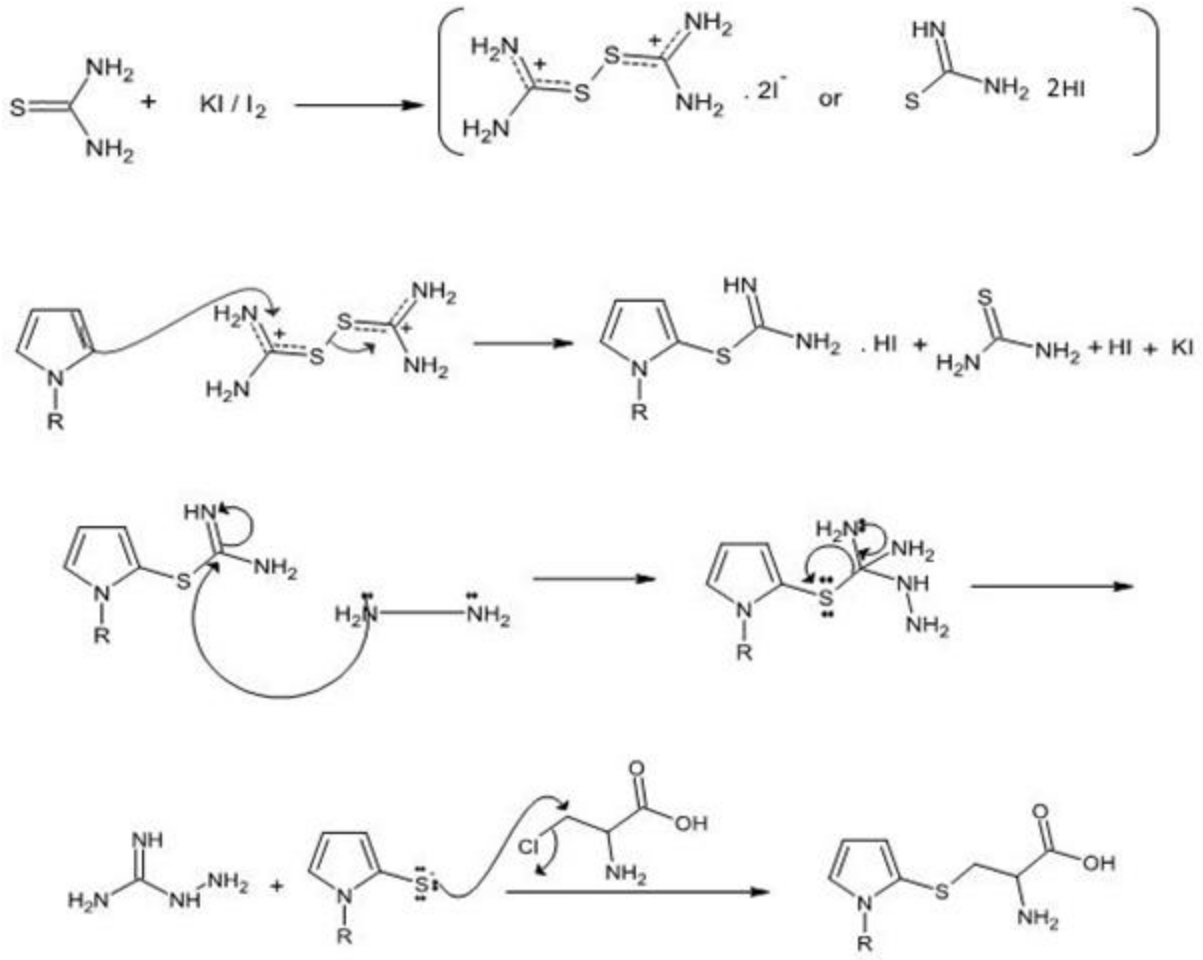


Figure 4.3 Mechanisms of synthesis of pyrrolyl cysteine. The mechanism was adopted from Rudyakova et al. (2008) with modification.

4.4 Stability and reactivity of the pyrrole compounds as educt

The pyrrole compounds used as educts in this work can be classified based on their yield and reactivity in each reaction as well as the contained pyrrole, N-methyl pyrrole, N-benzyl pyrrole and N-boc pyrrole.

Generally, the more electron-rich the compounds were, the more reactivity rate they showed. The above classification, however, follows a reverse order when the stability of these compounds is considered: S-(N-benzylpyrrol-2-yl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, and S-(2-pyrrolyl)cysteine. Therefore, the synthesis of S-(N-benzylpyrrol-2-yl)cysteine is more feasible due to the high stability, ease of reactivity, oxidization, and purification (using preparative HPLC).

Regarding the structural similarity between S-(N-methylpyrrol-2-yl)cysteine, S-(2-pyrrolyl)cysteine, and the small methyl group linked to nitrogen, it should not have a negative effect on the alliinase activity. The high instability of the two latter compounds makes it nearly

impossible to examine them in sufficient purity using HNMR, CNMR, MS and IR. They readily oxidize at room temperature and polymerize to a semi-liquid, sticky, brown viscous compound. The oxidation and analysis are negatively affected even under argon atmosphere, vacuum, light, and temperature protection.

To achieve S-(2-pyrrolyl)cysteine S-oxide from S-(N-benzylpyrrol-2-yl)cysteine S-oxide, the benzyl group has to be removed. Removing the benzyl group from pyrrole ring is rather difficult (for details, see methods). Therefore, N-Boc-pyrrole was analyzed, not resulting in the desired product for the following reasons: i) the protecting Boc-group has large dimensions, and its steric hindrance inhibits it from being placed on position 2 of the pyrrole ring; and ii) additionally, this reaction was not conducted in position 3 due to electron deficiency in the pyrrole ring. The Boc group converted the electrophilic compound to nucleophile, which could be explained by its electron density (**Figure 4.4**).

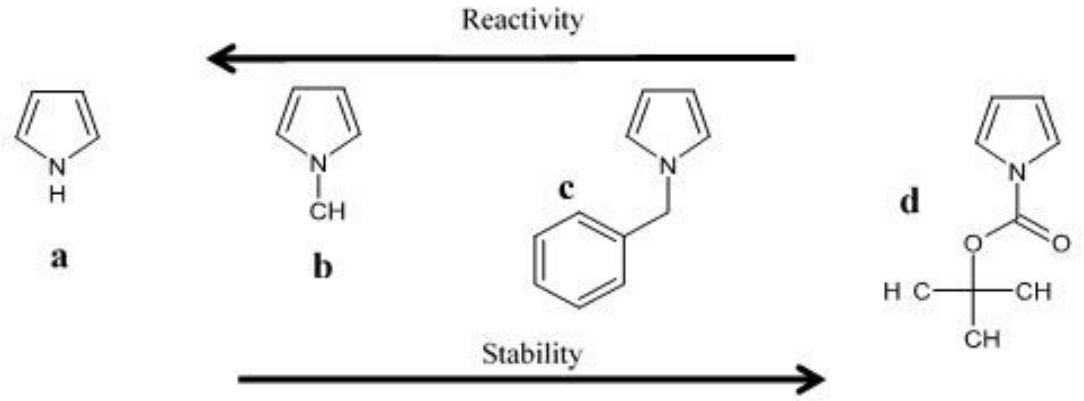


Figure 4.4 Comparison of stability and reactivity of primary compounds used to synthesize the products: a) pyrrole; b) N-methyl pyrrole; c) N-benzyl pyrrole; and d) N-Boc pyrrole.

4.5 Oxidation

Various unselective oxidations were applied to oxidize the synthesized sulfur compounds (for details, see methods). The oxidation of S-(N-benzylpyrrol-2-yl)cysteine was more straightforward than that of the other compounds. We could have risked the production of sufodioxide instead of sulfoxide if the oxidative compound had not been gently applied to the reaction mixture.

The steric hindrance of the benzyl group in oxidation of S-(N-benzylpyrrol-2-yl)cysteine may be disadvantageous for the following reasons: i) a strong oxidative like H_2O_2 in acetic acid needs to be used; ii) the reaction lasts approximately 15-18 h; and iii) removing the benzyl group in order to achieve the desired product is difficult. However, there are some advantages: i) this

compound is more stable than S-(N-methylpyrrol-2-yl)cysteine and S-(2-pyrrolyl)cysteine; and ii) the high steric hindrance of the benzyl group may result in a diastereoselective oxidative reaction and the inhibition of the racemates formation. The oxidation often takes place on the free side of the molecule, due to the steric hindrance on the opposite side. Thus, the yield product will be S or R, though its configurations remain unknown.

Acetic acid and methanol have similar solubility coefficiency; however, only acetic acid was used for oxidation reaction. The oxidation of methanol to formic acid via H_2O_2 and its competition with the educts can underestimate the equivalent calculations. In general, despite some difficulties in the oxidation of the two other products, S-(2-pyrrolyl)cysteine and S-(N-methylpyrrol-2-yl)cysteine, as a result of high instability different oxidative compounds were used (for details, see methods). The molecular weight of the desired compound often appeared two mass units lighter than the expected one (Appendices 40 and 42). Two explanations are possible: sulfur theoretically tends to oxidization rather than dehydrogenation, but the dehydrogenation of the compound and the formation of a double bond would be more likely to occur through MS analysis.

4.6 Hydrogenation

4.6.1 Hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine S-oxide

It is necessary to remove the benzyl group from S-(N-benzylpyrrol-2-yl)cysteine-S-oxide through hydrogenation to achieve the desired product S-(2-pyrrolyl)cysteine S-oxide. It is assumed that the catalyst is poisoned by sulfur and the reaction follows a different direction. Different types of sulfur such as H_2S thiol and thioether are poisonous to the catalytic reaction (Grove, 2003). Sulfur poisoning can happen in two reaction pathways, although bulk sulfidation is more likely than the surface sulfidation (Dunleavy, 2006). The mechanisms can be described as follows: if methanol as a reaction solvent is not fully dried, the water can cause sulfur poisoning, similar to the reaction of thioethers with catalyzer Pd (Traeger et al., 2012).

This hypothesis may be supported by the existence of a sulfur dioxide compound (2-amino-3-[(1-benzyl-1H-pyrrol-2-yl)sulfonyl]propionic acid) and an S-(N-benzylpyrrol-2-yl)cysteine compound in reaction mixture. To confirm the occurrence of sulfur poisoning in the reaction mixture, N-benzyl pyrrole was hydrogenated under similar conditions during the next step of the experiment (for details, see methods). The removal of the benzyl group from the pyrrole ring was successfully completed (**Appendix 19**). It may be possible to reduce the double bonds

in the pyrrole ring, if the pressure is increased up to 4 bar (Adkins and Coonradt, 1941; Dress et al., 2005 [patent WO/2005/103003]).

4.7 Comparison of the sulfur compounds

The synthesis was implemented using the sulfur compounds base and the mechanisms of nucleophilic substitution reaction, in which the thiol group is known as one of the best nucleophiles and 3-chloro-L-alanine with a very suitable leaving group like chloride. According to the SN_1 or even SN_2, the reaction has to be alkalinized by using, for example, KOH and heated in a water-free condition.

2-thiophenethiol, 2-mercaptoimidazole, 2-mercapto-1-methylimidazole and 2-thiazoline-2-thiol (as nucleophiles) were used as primary educts in the synthesis of the five-ring heteroaromatic cysteine sulfoxides as homologous substances. Unexpectedly, apart from 2-thiophenethiol, the other compounds did not result in the desired products. Two possible reasons can be given for this: i) alkaline, (e.g. KOH) probably competes with thiol, which enables the production of thionamide (Chan et al., 2014; **Figure 4.5**); ii) the existence of ethanolate ($Eto^{\ominus}$) as a solvent competes with thiol resulting in ether as a side product (**Figure 4.6**). Two solutions were tested for each of these problems: i) using potassium t-butilate instead of KOH to avoid the deformation of thionamide since high steric hindrance of potassium t-butilate inhibits its incorporation in the reaction; ii) using an aprotic solution like DMF instead of ethanolate (interrupting the solubility of 3-chloro-L-alanine). However, these solutions were not successful.

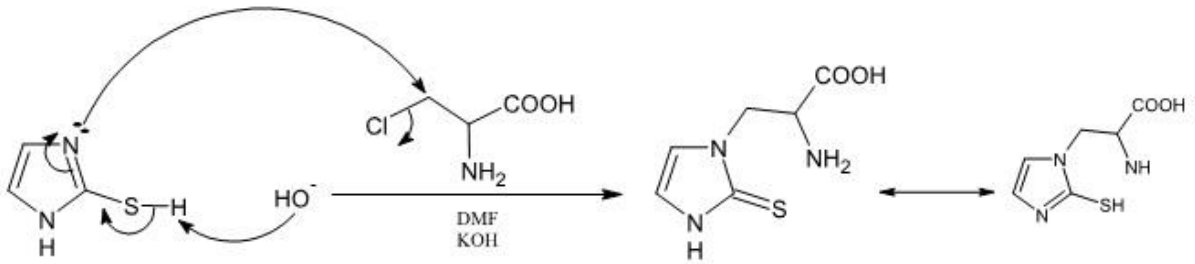


Figure 4.5 The formation mechanisms of thioamide.

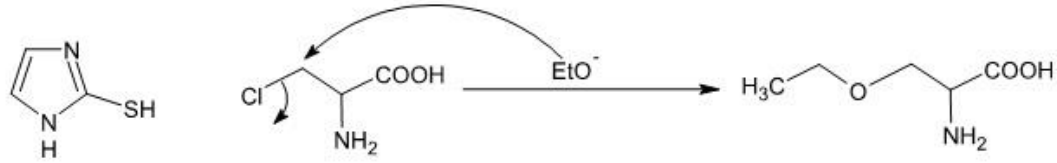


Figure 4.6 Competition of ethanolate with thiol in SN reaction.

4.8 H-NMR compression of compound S-(N-benzylpyrrol-2-yl)cysteine S-oxide with other aromatic and aliphatic sulfoxides

This study, its findings, and the literature review cited aimed to introduce an acceptable model for cysteine sulfoxides compounds. In ABX-splitting (**Figure** 4.5), Keusgen (1999) stated that the chemical shifts of protons in the methylene group differ by 0.25 ppm in (+)alliin and (+)propiin, although the difference between these two proton signals declines to 0.07 ppm in their (–)isomers. This scenario can be explained by the similarity to γ-glutamylmarasmin (Van den Broek et al., 1987). The methylene hydrogens can be presented in a triplet form instead of dd (Keusgen, 1999). γ-Glutamylmarasmin shows coupling constants (*J*) between the methylene and methin hydrogengs as J_{Ax} = 5.5/7 and J_{Bx} = 7/8.5 Hz for Ss–(+)isomer, and J_{Ax} = 3.5/4 and J_{Bx} = 8.5/10.5 Hz for Rs–(–)isomer (Kubota et al.,1998; Kubec & Velíšek, 2007). In (2-pyrridinyl)cysteine S-oxide, the difference between the two diasteropic proton signals was 0.27 ppm for (+)–isomer and 0.16 ppm for (–)isomer. In addition, coupling constants were calculated as 5.7/7.4 and 2.9/9.5 Hz for both positive and negative isomers, respectively (Mielke, 2015-doctoral thesis).

Unselective oxidation methods were implemented for the synthesis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide, since the synthesis of either positive or negative isomers was not the purpose of the present dissertation. As expected, the oxidized form (racemate) of the S-(N-benzylpyrrol-2-yl)cysteine-S-oxide and S-(2-thienyl)cysteine-S-oxide was present due to an unselective reaction. However, a large volume of the benzyl group may follow stereo selective reaction by blocking molecule from one side and form pure isomers (+ or -).

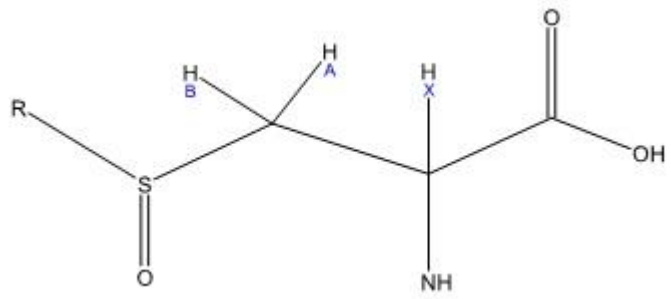


Figure 4.7 Molecular structure of aliphatic part of cysteine sulfoxide.

The chemical shift difference between the diastereotopic hydrogens (Ha and Hb) was calculated and compared with the similar products in the literature. S-(N-benzylpyrrol-2-yl)cysteine-S-oxide showed the chemical shift difference of about 0.31 ppm between protons of the methylene groups and a coupling constant of J_{Ax} = 8 and $J_{B\,x}$= 4.5 Hz. In S-(2-thionyl)cysteine-S-oxide the

chemical shift differed by 0.26 ppm and the coupling constant was estimated to be J_{Ax} = 2.52 and J_{Bx} = 9.62. As a result, the formation probability for (+)–S-(N-benzylpyrrol-2-yl)cysteine-S-oxide and (+)–S-(2-thionyl)cysteine-S-oxide increases (**Table 4.1**).

Table 4.1. Comparison of diastereotopic methylene hydrogen signals of ^{1}H-NMR of cysteine sulfoxides from literature including this work.

Reference	**δH_a-δH_b (ppm)**	**coupling constant J_{Ax}/J_{Bx} [Hz]**
SSRC-Alliin [Keusgen 1999]	0.24	6.0/7.7
RSRC-Alliin [Keusgen 1999]	0.07	4.0/7.9
*SS**R***C-Propiin [Keusgen 1999]	0.25	5.7/6.6
RSRC-Propiin [Keusgen 1999]	0	5.7/8.0
SSRC-Petiveriin [Kubec et al. 2001]	0.29	6.9/6.77
RSRC-Petiveriin [Kubec et al. 2001]	0.1	3.6/8.4
RSRC-Marasmin [Kubota et al. 1998]	0.25	7.3/6.6
*SS**R***C-Marasmin (als -glutamyl-Derivat) [van den Broek *et al.* 1987]	0	3.9/10.4
RSRC-PhCSO [Mielke. 2015- doctoral thesis]	0.15	6.0/6.6
*SS**R***C-PhCSO [Mielke. 2015- doctoral thesis]	0.07	3.5/8.5
RSRC-2-PyCSO [Mielke. 2015- doctoral thesis]	0.27	5.7/7.4
SSRC-2-PyCSO [Mielke. 2015- doctoral thesis]	0.16	2.9/9.5
S-(N-benzylpyrrol-2-yl)cysteine-S-oxide [this work]	0.31	8/4.5
S-(2-thienyl)cysteine-S-oxide [this work]	0.26	2.52/9.62

4.8.1 ^{1}H-NMR comparison of diastereotopic methylene hydrogen signals in the non-oxidized compounds

The comparison of aliphatic or cysteine parts of the non-oxidized compounds revealed that these compounds are different both in coupling constants and chemical shifts. The highest and lowest difference of the chemical shift of the diastereotopic methylene hydrogens were observed in S-(N-methylpyrrol-2-yl)cysteine and S-(2-pyrrolyl)cysteine, respectively (**Table 4.2**).

Table 4.2. Comparison of diastereotopic methylen hydrogen signals of ^{1}H NMR of the non-oxidized compounds.

Compound	**δH_a-δH_b (ppm)**	**J_{Bx}**	**J_{Ax}**
S-(2-pyrrolyl)cysteine	0.25	8.70	3.66
S-(N-benzylpyrrol-2-yl)cysteine	0.43	10.53	3.21
S-(2-thienyl)cysteine	0.45	9.80	3.60
S-(2-pyridinyl)cysteine (Milke, 2015-doctoral thesis)	0.54	md	Md
S-(N-methylpyrrol-2-yl)cysteine	0.66	8.70	5.40

4.9 Biosynthetic pathway of S-(3-pyrrolyl)cysteine sulfoxide

Vogt (2008, doctoral thesis) assumed that proline and ornithine are the primary compounds that convert to pyrrole-2-carboxylate.

The glutathione linked to the pyrrole ring in position 3 was oxidized, and the gamma-glutamyl group was removed using gamma-glutamyl transferase and converted to S-(3-pyrrolyl)cysteine S-oxide (**Figure** 4.8).

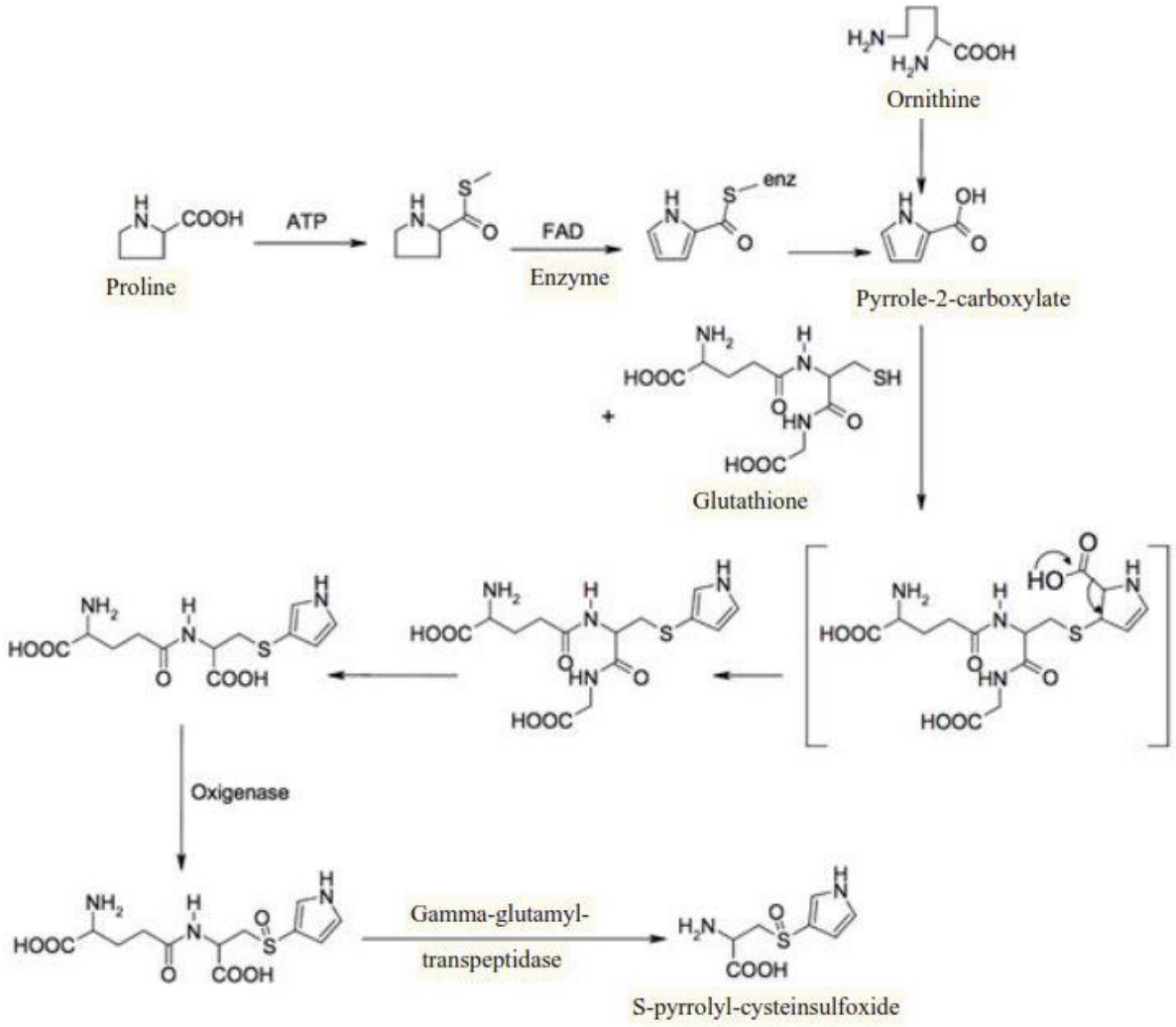


Figure 4.8 Possible biosynthesis pathway of S-(3-pyrrolyl) cysteine sulfoxides (Vogt, 2008-doctoral thesis).

4.10 Biosynthetic pathway of S-(2-pyrrolyl)cysteine sulfoxide

It is presumed that acetate and L-serine have a large effect on the biosynthesis pathway of S-(2-pyrrolyl)cysteine sulfoxide. Serine-kinase catalyzed the conversion of L-serine to L-serine monophosphate. The reaction was followed by linking oxaloacetate produced from acetate in the citric acid cycle to L-serine mono phosphate using a hypothetical protein.

The function of this protein is unknown (Siebenberg et al., 2011). L-serine monophosphate was further converted to the precursor compound pyrrole-3-carboxylate or pyrrole-2-carboxylate under the reaction of decarboxylase and the dehydrogenase enzymes. To achieve the desired product S-(2-pyrrolyl)cysteine S-oxide, two possible pathways can be proposed: i) In nucleophilic substitution reaction (S_N-Ar), glutathione attacks the aromatic ring and converts to the intermediate substance under the rearomaticity and decarboxylation. This is catalyzed by oxygenase. Gamma-glutamyl transferase conducts the reaction to produce the desired product; ii) Cysteine kinase leads to the conversion of L-cysteine to L-cysteine mono phosphate and its linkage to the pyrrole ring in position 2 under an electropholic substitution reaction in aromat S_E-Ar (**Figure 4.9**).

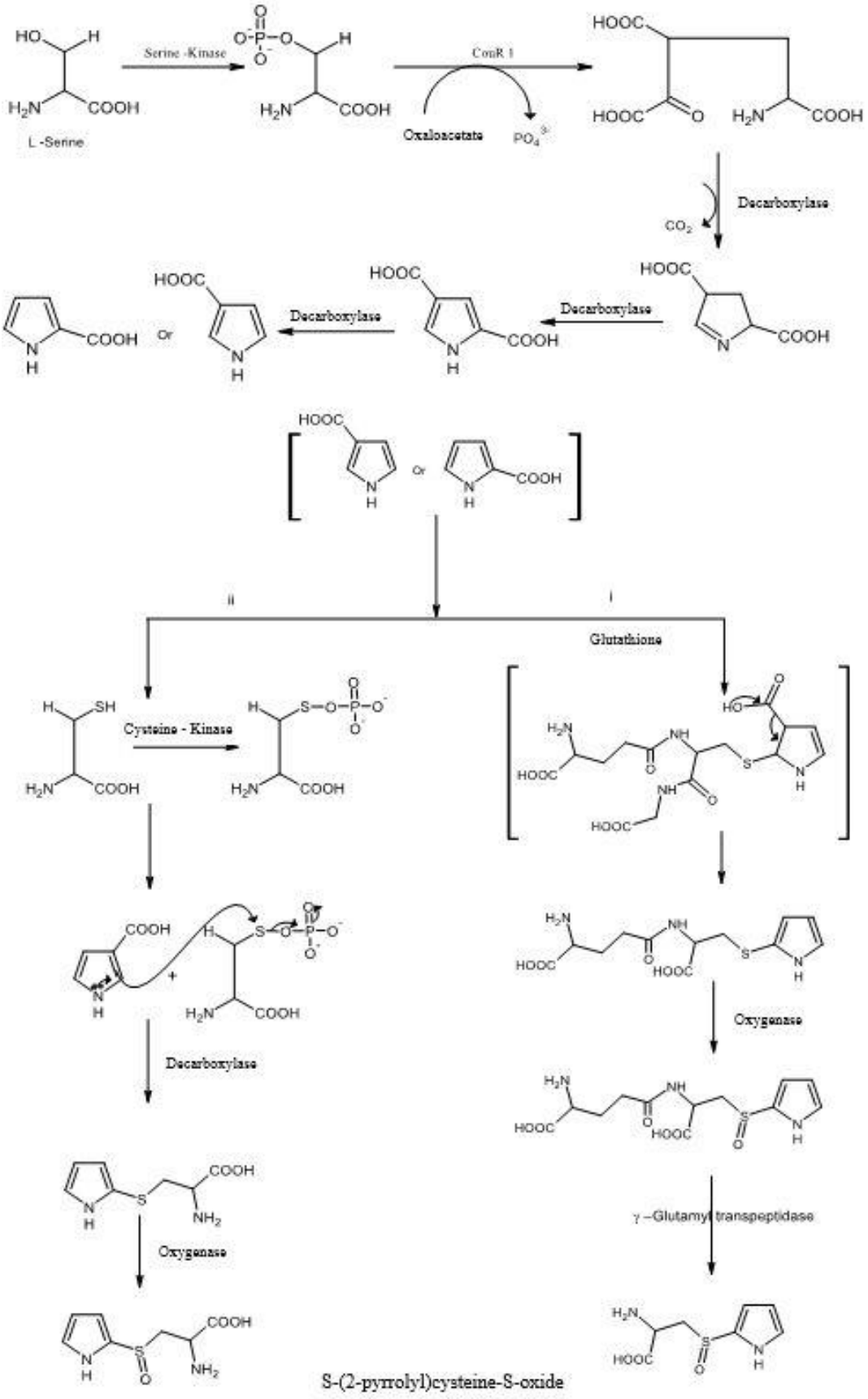


Figure 4.9 Hypothetical biosynthesis pathway of S-(2-pyrrolyl)cysteine sulfoxides (after Vogt, 2008-doctoral thesis and Siebenberg et al., 2011).

4.11 Enzymatic digestion

Enzymatic digestion was performed on the six synthesized products in either forms of sulfide and sulfoxide, using alliinase from *A. sativum* (Krest & Keusgen, 1999b).

4.11.1 The compound at *m/z* 136 $[M+H]^+$

Under negative control, two different compounds with the molecular formula of $C_9H_{14}N$ and with pyridine content were detected. Ammonium salt, which was probably produced due to buffer contamination, could be one of the compounds (**Figure 4.10iiia**). It can link to other compounds such as allicin, ajoen, or S-thiophene-2-yl thiophene-2-sulfinothioate (**Figure 4.10i**). A pyridoxal-phosphate derivative, considered an important co-enzyme for alliinase (Percudani & Preacchi, 2003), is the second possible compound (**Figure 4.10iiib**).

4.11.2 Enzymatic digestion of S-(2-thienyl)cystein-S-oxide

The test was comprehensively conducted under positive and negative controls for the compound S-(2-thienyl)cystein-S-oxide. Alliin was used in the positive control. In the positive control, allicin and ajoene were identified as ammonium salts. Under positive control conditions, the reaction of alliinase with S-(2-thienyl)cystein-S-oxide resulted in the expected products: disulfide-S-oxide (**Figure 4.10i**) as well as its oxidation product disulfide-S-S´-dioxide, which is also known as the ammonium salts (**Figure 4.10ii**).

4.11.3 Enzymatic digestion of the synthesized pyrrole compounds

The reaction of alliinase with pyrrole compounds, including S-(2-pyrrolyl)cysteine, S-(N-methylpyrrol-2-yl)cysteine, S-(N-benzylpyrrol-2-yl)cysteine, and S-(N-benzylpyrrol-2-yl)cysteine-S-oxide showed a high degree of compound similarity. These products, however, are mainly identified as side-products because of the high reactivity and instability of thiosulfinates (Block et al., 1993). The reaction of alliinase with any of these four compounds may expose the compounds with different molecular weights to molecular charge ratio as follows: at *m/z* 294 $[M+H]^+$, corresponding to the molecular formula of $C_{14}H_{16}NS_3$ (**Figure 4.10iv**); at *m/z* 231 $[M+H]^+$, corresponding to the molecular formula of $C_8H_{11}N_2S_3$ (**Figure 4.10v**) or $C_8H_{11}N_2O_2S_2$ (**Figure 4.10vi**); at *m/z* 266 $[M+H]^+$ corresponding to the compounds with the molecular formula of $C_{11}H_{12}N_3OS_2$ (**Figure 4.10vii**) or $C_9H_{16}NO_2S_3$ (**Figure 4.10viii**).

4.11.4 Enzymatic digestion of S-(2-thienyl)cysteine

Although the reaction of alliinase with S-(2-thienyl)cysteine resulted in several side products, the existence of the compound shown at *m/z* 299 $[M+H]^+$ with the molecular formula of $C_{11}H_{11}N_2O_2S_3$ (**Figure 4.10ix**) is more realistic.

4.11.5 Conclusion and summary of enzymatic tests

In summary, it can be concluded that of the six synthesized compounds in this research, only the reaction of S-(2-thienyl)cystein-S-oxide as a heteroaromatic cystein sulfoxide with the existing alliianse in *A. sativum* resulted in the production of the expected compounds, disulfide-S-oxide (**Figure 4.10i**) and disulfide-S-S´-dioxide (**Figure 4.10ii**). In contrast, the reaction of other five synthesized compounds with alliinase produced by-products other than the expected compounds. In (3.10.2) the molecular formulas of these compounds together with their possible molecular structures were described.

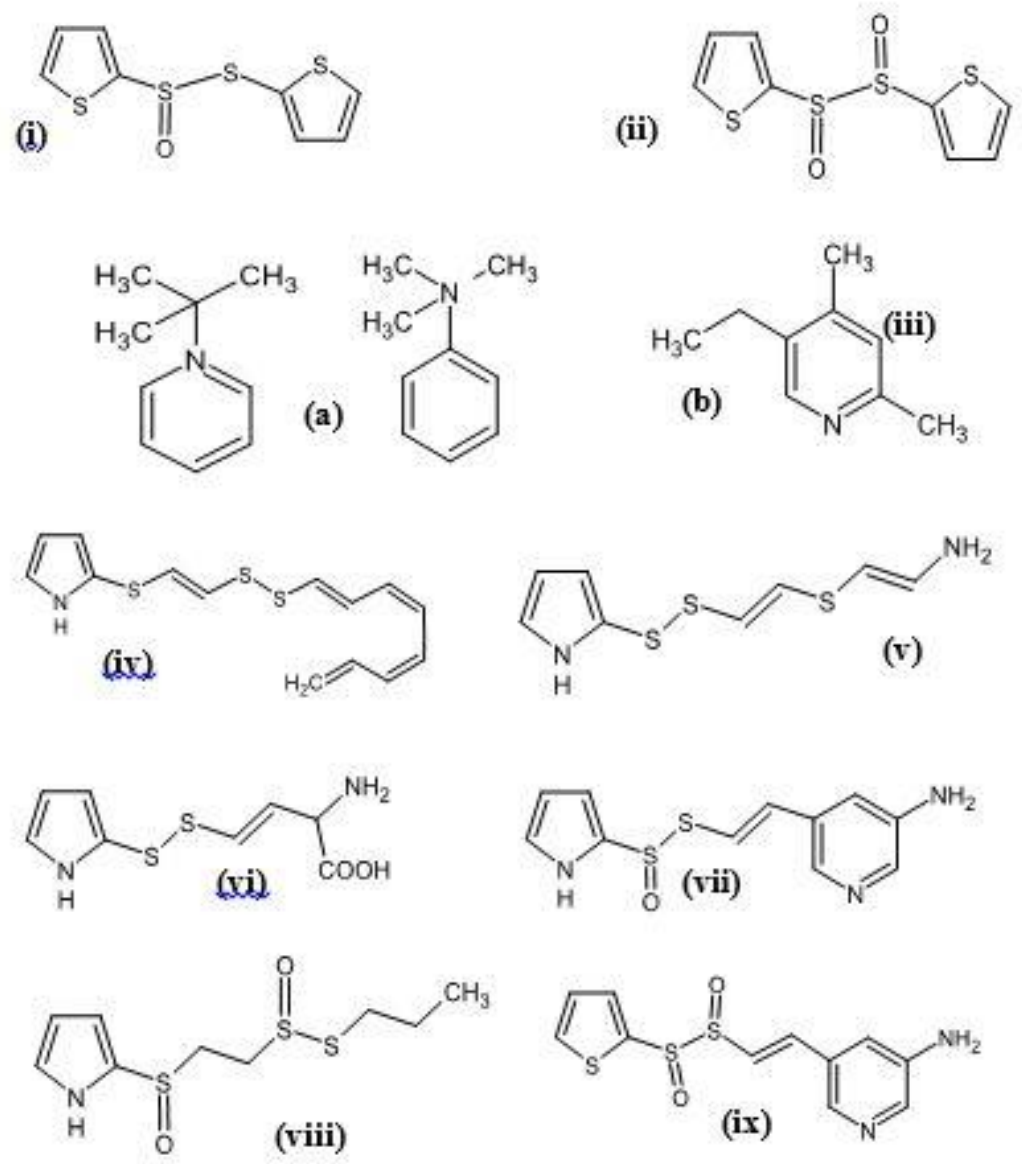


Figure 4.10 Molecular structure of (i) S-(2-thienyl)thiophene-2-sulfinothioate; (ii) 2,2′-(1,2-dioxido-1 λ4, 2 λ4-disulfane-1,2-diyl)dithiophene; (iii) a: 1-*tert*-butylpyridinium (left), *N*,*N*,*N*-trimethylanilinium (right); b: 5-ethyl-2,4-dimethylpyridine; (iv) 2-({(*E*)-2-[(1*E*,3*Z*,5*Z*)-octa-1,3,5,7-tetraen-1-yldisulfanyl]ethenyl}sulfanyl)-1 *H*-pyrrole; (v) (*E*)-2-{[(*E*)-2-(1*H*-pyrrol-2-yldisulfanyl)ethenyl]sulfanyl}ethenamine; (vi) (3*E*)-2-amino-4-(1*H*-pyrrol-2-yldisulfanyl)but-3-enoic acid; (vii) S-[(*E*)-2-(5-aminopyridin-3-yl)ethenyl] 1 *H*-pyrrole-2-sulfinothioate; (viii) S-propyl 2-(1*H*-pyrrol-2-ylsulfinyl)ethanesulfinothioate; (ix) 5-{(E)-2-[1,2-dioxido-2-(thiophene-2-yl)-1λ4,2λ4-disulfan-1-yl]ethenyl}pyridin-3-amine.

Five possible explanations for the synthesis of these unexpected products are as follows: i) impurities in the synthesis and the contamination of the buffer; ii) instability of thiosulfinates such as allicin; iii) cysteine sulfoxides are the specific substrates for aliinase; iv) substrate specificity of alliinase; v) various enzymatic activity on (+) and (-) substrate isomers.

4.11.5.1 Impurity during synthesis and contamination of buffer

The instability of the compounds S-(2-pyrrolyl)cysteine and S-(N-methylpyrrole-2yl)cysteine in the presence of light, room temperature, and oxygen increases the rate of side product formation before and after alliinase test due to the impurity of synthesized substrates. Factors like polymerization and oxidation, or recombination of these undesired products with the primary substances or other side products, can result in unexpected side products in the reaction. Alternatively, buffer contamination can cause side products like the compound with the molecular weight of 135 g/mol, which was observed in the negative control.

4.11.5.2 Instability of thiosulfinates like allicin

Thiosulfinates, which are also highly instable in the presence of high temperature, light and oxygen, are involved in various chemical reactions such as radicalic addition or substitution, α-β-elimination, condensation, [3,3] and [2,3]-sigmatrope rearrangement, (i.e. from sulfoxide-accelerated thio and dithio claisen-rearrangement) and cycloaddition with thiocarbonylcysteine (Block et al., 1993). Thiosulfinate including alliicin can rearrange to sulfur dioxide or diallyl-mono-, di-, and trisulfides at RT (Brondnitz et al., 1971).

Radical substitutions or additions under the effect of light and temperature are among the main factors contributing to the formation of the side products with respect to pyrrole compounds (**Figure** 4.11) (Block et al., 1993).

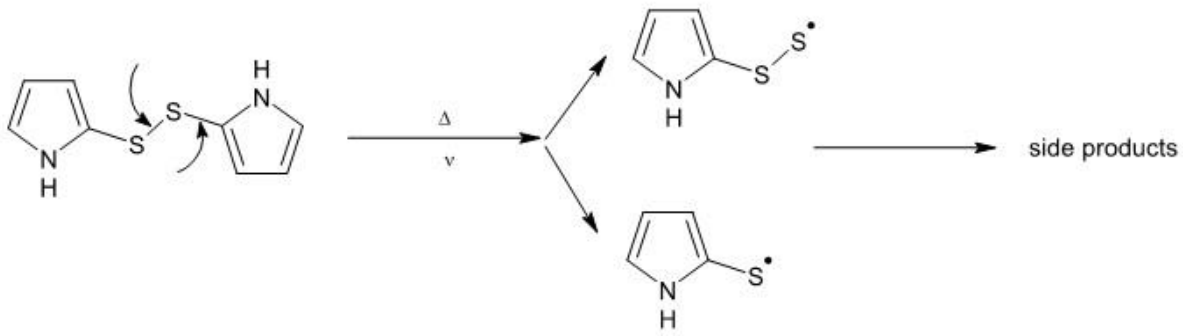


Figure 4.11 Formation of the side prodcuts due to the radicalic reaction.

Apart from allicin in the positive control and S-thiophene-2yl thiophene-2-sulfinothiolate (**Figure 4.10 i**), the expected product of the reaction of S-(2-thienyl)cysteine-S-oxide on aliinase, the other identified substances are considered side products.

4.11.5.3 Cysteine sulfphoxides are specific substrates for aliinase

The alliinase reaction with sulfur or sulfur dioxide compounds did not result in the production of the expected compounds, as it is a naturally specific enzyme for cysteine sulfoxide compounds. The sulfoxide group (S=O) plays a significant role in allinase enzyme site (Kuettner et al., 2002). It was expected to oxidize the compounds S-(2-pyrrolyl)cysteine or S-(N-methylpyrrol-2-yl)cysteine to sulfoxide during the reaction because of its high instability against light, room temperature, and oxygen. The absence of a reaction of the substrate S-(N-benzylpyrrole-2-yl)cysteine-S-oxide with alliinase can be explained by the following hypothesis: the large benzyl group connected to the pyrrole ring can considerably reduce the possibility of coupling S-(N-benzylpyrrole-2-yl)cysteine-S-oxide with allinase in the enzyme site and as a result, the desired product will not be obtained.

4.11.5.4 Substrate specificity of alliinase

Considering the reactivity of substrates by alliinase, the alkyl cysteine sulfoxides are less reactable than the alkenyl cysteine sulfoxides (Krest, 1998-doctoral thesis).

Similar substrates were used for the enzymatic digestion in *A. sativum* (alliinase used in this test) and alliinase of *A. macleanii* of subgen. *Melanocrommyum* (Vogt, 2008-doctoral thesis). **Table** 4.3 displays the results of this work along with the previous reports discussed here. The highest enzymatic activity rate in *A. sativum* was shown to be for alliin, while methiin substrated with enzymes activity of about 20%, propiin with about 36% and butiin with about 12% (Knobloch et al., 1991; Krest et al., 2000).

By contrast, the alliinase extracted from *A. macleanii* containing pyrrolyl cysteine sulfoxide, as heteroaromatic cysteine sulfoxide, showed no activity to methiin and butiin and only 30% activity towards alliin. The maximum activity reported was that of the heteroaromatic pyrrole ring in cysteine sulfoxides (Vogt, 2008-doctroral thesis). Alliinase extracted from *A. stipitatum* appeared with low activity for pyrridinyl cysteine sulfoxide and even lower for S-pyrridinyl cysteine N-oxide (Mielke, 2015-doctoral thesis). In agreement with previous studies, our work shows that the substrate specificity is significant in enzymatic activity of *A. sativum* with the synthesized substrates.

4.11.5.5 Various enzymatic activities on (+) and (-) substrate isomers

(+)- Isomers were found in plants (Koch & Lawson, 1996). Stereoisomers (+) or (-) largely affect either the reaction velocity with the enzyme or its specificity. It is more likely that (+)-isomers react with alliinase and that the reactivity of negative isomers is less than that of positive ones (e.g. by alliin: Krest & Keusgen, 1999b). However, the (-)-isomer from S-(2-pyrridinyl)cysteine-S-oxide is an exception and is more effective than the (+) isomer in the reaction with alliinase from *A. sativum* (Mileke, 2015-doctoral thesis).

Apart from S-(N-benzylpyrrol-2-yl)cysteine-S-oxide which probably contains (+)- isomers instead of (-)-isomers (based on the comparison made in the section 4.8), the isomer types of other synthesized substrates in this study are unknown. In addition, the benzyl group linked to the nitrogen of pyrrole prohibits the enzyme-substrate reaction at the binding site. Reactivity of each substrate not only depends on the compatibility of the enzyme site but also on the side chain of the adjacent amino acid (Vogt, 2008-doctoral thesis).

Table 4.3 Comparison activity of alliinase on synthesized substrates with literature.

	Relative activity of enzyme				
Cysteine sulfoxide derivatives	***A. macleanii* (%)**	***A. sativum* (%)**		***A. stipitatum***	***A. sativum***
	Vogt, 2008-doctoral thesis	**Knobloch**	**Krest**	**Mielke, 2015-doctoral thesis**	**This work**
Pyrrole	100.00	unstudied	unstudied		unstudied
Methiin	0.20	21	20		unstudied
Alliin	30.30	100	100		unstudied
Propiin	36.03	36	6		unstudied
Butiin	0.13	unstudied	12		unstudied
Pytidin	42.42	unstudied	unstudied		unstudied
Benzyle	16.39	unstudied	unstudied		unstudied
Furan	42.50	unstudied	unstudied		unstudied
Thiazol	72.95	unstudied	unstudied		unstudied
S-(2-pyrridinyl)cysteine-S-oxide				Positive	
S-(2-pyrridinyl)cysteine-N-oxide				Positive	
Phenylcysteine-S-oxide				positive	
S-(2-pyrrolyl)cysteine					insignificant
S-(N-methylpyrrol-2-yl)cysteine					insignificant
S-(N-benzylpyrrol-2-yl)cysteine					insignificant
S-(N-benzylpyrrol-2-yl)cysteine S-oxide					insignificant
S-(2-thienyl)cysteine					insignificant
S-(2-thienyl)cysteine-S-oxide					positive

5. Summary

Cysteine sulfoxides are important secondary compounds in *Allium sativum* and *A. cepa*, and have been known as substrates specific for enzyme alliinase. Different types of cysteine sulfoxides have been identified in many of *Allium* species, of which methiin, alliin, propiin, butiin and marasmin are some of the best-known and best-studied ones.

S-(2-pyrrolyl)cysteine-S-oxide is one of heteroaromatic cysteine sulfoxides containing a pyrrole ring. It has been largely reported in *A. rosenorum*, *A. macleanii*, *A. giganteum,* and *A. carolanianum* of subgen. *Melanochrommyum*. S-(2-pyrrolyl)cysteine-S-oxide is converted to the red pigment or 2,2′-epidithio-3,3′-dipyrrole under alliinase reaction. To understand the molecular structure, pharmacology, and medicinal usage of this interesting pigment, the synthesis of this type of cysteine sulfoxides and their homologues with enough similar characteristics is necessitated.

In this work, six compounds were synthesized either as a sulfoxide or sulfide that contained: S-(N-benzylpyrrol-2-yl)cysteine (47% yield); S-(N-methylpyrrol-2-yl)cysteine (23% yield); S-(2-pyrrolyl)cysteine (35% yield); S-(2-thienyl)cysteine (93% yield); S-(N-benzylpyrrol-2-yl)cysteine-S-oxide (30% yield) and; S-(2-thienyl)cysteine-S-oxide (48% yield). The first four compounds were synthesized as sulfides and two latter as cysteine sulfoxides. The first three abovementioned products were synthesized using heteroaromatic N-benzyl pyrrole, N-methyl pyrrole, and pyrrole in combination with thiourea and KI/I_2, which was followed by alkalization of the reaction mixture. 3-Chloro-L-alanine, as the key primary compound, was added to the mixture and heated up to 100˚C for 2 h under argon protection. Final products, if stable, could be purified using preparative-HPLC or re-precipitated using diethylether dissolved in low amounts of methanol. A different method was conducted to synthesize S-(2-thienyl)cysteine, as compared to method used to synthesize the first three products. A nucleophilic substitution reaction was carried out between sulfur compounds (as nucleophile) and 3-chloro-L-alanine.

The next step in obtaining cysteine sulfoxides was to oxidize the synthesized products. Out of six products, S-(2-thienyl)cysteine and S-(N-benzylpyrrol-2-yl)cysteine were successfully oxidized using H_2O_2 in water or acetic acid, resulting in S-(2-thienyl)cysteine-S-oxide and S-(N-benzylpyrrol-2-yl)cysteine-S-oxide. If they were stable, the purification of these products was performed using preparative HPLC. The oxidation of the remaining products (S-(N-

benzylpyrrol-2-yl)cysteine, S-(2-thienyl)cysteine, S-(N-methylpyrrol-2-yl)cysteine and S-(2-pyrrolyl)cysteine), was not possible because of their high instability and polymerization against light, oxygen, and temperature. Additionally, weak oxidants, such as MMPP, were unable to oxidize these compounds.

Lastly, an enzymatic test was conducted to estimate the reactivity of all the synthesized products to alliinase. To adjust the optimum pH for the enzyme alliinase, alliinase buffer was added to the products, which were dissolved in water. The reaction was followed by adding cyclohexane (organic phase) to the reaction mixture and incubating the mixture for 2 h: in the end, the organic phase was separated, dried with $MgSO_4$, and evaporated. Alliin was used as positive control.

S-(2-thienyl)thiophene-2-sulfinothioate was successfully separated and identified using HPLC-MS.

- **Zusammenfassung**

Cysteinsulfoxide sind wichtige sekundäre Metabolite in *Allium sativum* und *A. cepa*, die als spezifisches Substrat für das Enzym Alliinase bekannt sind. Verschiedene Arten von Cysteinsulfoxiden wurden in zahlreichen *Allium*-Spezies identifiziert, von denen Methiin, Alliin, Propiin, Butiin und Marasmin zu den bekanntesten und meistuntersuchten Verbindungen gehören.

S-(2-Pyrrolyl)cystein-S-oxid ist ein heteroaromatisches Cysteinsulfoxid, das einen Pyrrolring enthält. Es wurde bisher in *A. rosenorum, A. macleanii, A. giganteum und A. carolanianum* aus der Untergattung *Melanochrommyum* gefunden. S-(2-Pyrrolyl)cystein-S-oxid wird durch die Alliinasereaktion in das rote Pigment 2,2'-Epidithio-3,3'-dipyrrol umgewandelt. Um die richtige Struktur, Pharmakologie und medizinische Verwendung dieses interessanten Pigments zu verstehen, ist die Synthese des Pyrrol-Cysteinsulfoxides und/oder seiner Homologen in ausreichenden Mengen erforderlich.

In dieser Arbeit wurden sechs Verbindungen entweder als Sulfoxid oder Sulfid synthetisiert: S-(N-Benzylpyrrol-2-yl)cystein (47 % Ausbeute); S-(N-Methylpyrrol-2-yl)cystein (23 % Ausbeute); S-(2-Pyrrolyl)cystein (35 % Ausbeute); S-(2-Thienyl)cystein (93 % Ausbeute); S-(N-Benzylpyrrol-2-yl)cystein-S-oxid (30 % Ausbeute) und S-(2-Thienyl)cystein-S-oxid (48 % Ausbeute).

Die ersten vier Verbindungen wurden als Sulfide und die beiden letzteren als Cysteinsulfoxide synthetisiert. Die ersten drei oben genannten Produkte wurden unter Verwendung von heteroaromatischem N-Benzylpyrrol, N-Methylpyrrol und Pyrrol in Kombination mit Thioharnstoff und KI/I2 synthetisiert, woraufhin eine Alkalisierung der Reaktionsmischung für die weiteren Schritte notwendig ist. 3-Chloro-L-alanin als primäre Schlüsselverbindung wurde synthetisiert, zu dem oben genannten Gemisch gegeben und zwei Stunden unter Verwendung von Argon als Schutzgas auf 100 °C erhitzt. Wenn das Endprodukt stabil war, konnte es entweder unter Verwendung präparativer HPLC oder durch Ausfällung unter Verwendung von Diethylether gereinigt werden.

Daneben wurde noch ein anderes Verfahren angewandt, um S-(2-Thienyl)cystein zu synthetisieren. Dazu wurde eine nucleophile Substitutionsreaktion zwischen Mercaptoverbindungen (als Nucleophil) und 3-Chloro-L-alanin (mit Chlor als Abgangsgruppe) durchgeführt.

Der nächste Schritt bestand darin, die synthetisierten Produkte zu oxidieren, um das Cysteinsulfoxid zu erhalten. S-(2-Thienyl)cystein und S-(N-Benzylpyrrol-2-yl)cystein wurden erfolgreich mit H_2O_2 in Wasser oder Essigsäure oxidiert, was zur Herstellung von S-(2-Thienyl)cystein-S-oxid und S-(N-Benzylpyrrol-2-yl)cystein-S-oxid führte. Bei ausreichender Stabilität konnte die Reinigung dieser Produkte unter Verwendung der präparativen HPLC durchgeführt werden. Die Oxidation der anderen Produkte (S-(N-Benzylpyrrol-2-yl)cystein,
S-(2-Thienyl)cystein, S-(N-Methylpyrrol-2-yl)cystein und S-(2-Pyrrolyl)cystein) war wegen der hohen Instabilität und Polymerisation durch Licht, Sauerstoff oder Temperatur unter den verwendeten Reaktionsbedingungen nicht möglich. Sogar einige schwache Oxidationsmittel wie MMPP waren nicht in der Lage, diese Verbindungen ohne Nebenreaktionen zu oxidieren.

Schließlich wurde eine enzymatische Umsetzung durchgeführt, um die Reaktion aller in der Arbeit synthetisierten Produkte mit dem Enzym Alliinase von *A. sativum* zu untersuchen. Um das pH-Optimum für das Enzym Alliinase einzustellen, wurde Alliinase-Puffer zu Produkten, die im Voraus in Wasser gelöst wurden, gegeben. Die Reaktionsmischung wurde durch Zugabe von Cyclohexan (als organische Phase) ergänzt und für zwei Stunden inkubiert. Es wurde die organische Phase abgetrennt, über $MgSO_4$ getrocknet und eingeengt. Alliin wurde als positive Kontrolle verwendet.
S-(2-thienyl)thiophene-2-sulfinothioate wurde erfolgreich getrennt und unter Verwendung von HPLC-MS identifiziert.

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7. Supplementary data

Appendix 1. List of chemicals used in this study. * indicates the reference substances; PRN stands for "pro re nata".

Solid chemicals	Supplier	Amount
(Boc-Cys-OH)2 ≥98% (CE)	Sigma-Aldrich	5 g
2-Mercapto-1-methylimidazole	TIC America	25 g
2-Mercaptoimidazole 98%	Sigma-Aldrich	1 g
2-Mercaptothiazoline	TIC America	25 g
2-Thiophene thiol ≥97%	TIC America	5 g
2-Thiophenesulfonyl chloride 96%	Sigma-Aldrich	1 g
3-Bromofuran 97%	Sigma-Aldrich	5 g
3-Mercapto-1,2,4-triazole 97%	TIC America	25 g
Allyl isocyanate 98% (cotanining BHI as stabilizer, 95%)	Sigma-Aldrich	10 g
Chlorotriisopropylsilane 97%	Sigma-Aldrich	1 g
Dimethylsulfoxid p.a.	Merck, Darmstadt	41 g
Hydrazine solution 35% in H2O	Sigma-Aldrich	10 g
Imidazole 99%	Sigma-Aldrich	100 g
Kaliumjodid 99.5%	Grussing	100 g
L-Cysteine 97 %	SAFC (Sigma-Aldrich), St. Louis (USA)	38 g
L-Serine*	TIC America	25 g
Magnesiumsulfat-Anhydrat reinst	Grüssing, Filsum	250 g
N-Benzylpyrrole 97%*	Sigma-Aldrich	5 g
N-Boc-pyrrole 98%	Sigma-Aldrich	25 g
N-Bromosuccinimide ≥98%	TIC America	20 g
N-Z-L-Serine benzyl ester 97%	Sigma-Aldrich	1 g
Natriumhydroxid 99%	Grussing	250 g
Salzsäure 32 %	Fisher Scientific, Schwerte	25 g
Silica gel (pore size 60 A, 70-230 mesh, 63-200 μm, for column)	Fluka or Macherey Nagel, Dueren, Germany	PRN
Thiourea, ReagentPlus® ≥99.0%	Fulka	100 g
Thiourea, ReagentPlus® ≥99.0%*	Sigma-Aldrich	100 g
Trifluoromethanesulfonic anhydride ≥98% (TFMSA)	TIC America	10 g
Trifluoromethanesulfonic anhydride 99%	Sigma-Aldrich	5 g
Triphenylphosphine	Fulka	25 g
Zirconium(IV) chloride ≥99.9%	Sigma-Aldrich	5 g

β-chloro-L-alanine*	TIC America	1-5 g
Liquid chemicals		
2,6-Lutidine ≥ 98%	TIC America	25 ml
3-Bromothiophene	Merck	50 ml
Acetone	Lenz-Chemie, Westerburg	2.5 L
Ameisensäure reinst	Riedel-de-Haen, Seelze	30 L
Ammoniak 25 %	Fisher Scientific, Schwerte	33 L
Deuterium oxide 99.8%	Roth	25 ml
Ethanol	Lenz-Chemie, Westerburg	2.5 L

Appendix 1. continued

Ethyl acetate 99.99%	Fischer	2.5 L
Ethylacetat	Fisher Scientific, Schwerte	2.5 L
Furan 99%	Sigma-Aldrich	100 ml
Hydrazin solution 35 wt. % in water	Sigma-Aldrich	100 ml
Isopropanol 99,5 %	Acros Organics (Thermo Fisher Scientific), Geel (Belgien)	2.5 L
Methanesulfonic acid, ≥99.5%	Sigma-Aldrich	5 ml
Methanol 99.99%	Fischer	PRN
Methanol analytical reagent grade	Fisher Scientific, Schwerte	2.5 L
n-Butanol	Merck, Darmstadt	1 L
n-Butyllithium solution 2.5 M in Hexan	Sigma-Aldrich	2 ml
N-Methylpyrrole 99%	Sigma-Aldrich	100 ml
N,N,N′,N′-Tetramethylethylenediamine ReagentPlus® ≥99%	Sigma-Aldrich	100 ml
Pyrrole 98%	Sigma-Aldrich	100 ml
Pyrrole reagent grade 99%	Sigma-Aldrich	25 ml
Sulfonyl chloride solution 1 M in methylene chloride	Sigma-Aldrich	100 ml
tert-Butyllithium solution 1.7 M in pentane	Sigma-Aldrich	100 ml
THF Hochschullieferung	BASF, Ludwigshafen am Rhein	2.5 L
Thionyl chloride ReagentPlus® ≥99.0%	Sigma-Aldrich	500 ml
Triethylamine ≥99.5%	Sigma-Aldrich	100 ml
Wasserstoffperoxid 35 %	Merck, Darmstadt	26 L

Appendix 2. List of device and relevant information used in this study.

Devices	Compartment detail/supplier	Parameter/modus
Bruker Alpha-P FT-IR	Bruker, Ettlingen, Germany	ATR-FTIR-Modus
UV	Shimadzu, Japan (UV-2401PC)	
NMR-Spektrometer	Jeol, Tokyo, Japan	JEOL-ECA 500
Shimadzu Prominence HPLC	Kyoto, Japan	Controller (CBM-20A) Pumpe (LC-20AT) Degaser (DGU-20As) UV/Vis-Detector (SPD-20A) Software (LC solution)
Merck-Hitachi HPLC	Kyoto, Japan	Interface (D-7000) Säulenofen (L-7360) Autosampler (L-7200) Peltier Sample Cooler (L-7200) Degaser (L-7612) Pumpe (L-7100) Software (D-7000 HSM)
HPLC-MS	Macherey-Nagel, Düren Pumpe (Shimadzu LC10ADvp)	 wavelenght (334 nm)

	UV-Detector (Shimadzu SPD10Avp) Autosampler (Shimadzu SILHTC) Column oven (CATO10ASvp) Q Trap (LC/MS/MS System (Applied Biosystems (Thermo Fis- her Scientific), Darmstadt)) Software (Analyst (Applied Biosystems (Thermo Fisher Scienti- fic), Darmstadt))	Injected volumes (20 µL) Equilibration time (20 min) Flow rate (0.25 mL/min) temperature in autosampler (10°C)

Appendix 2. continued

Q-Trap	Ion source (Turbo Spray) Measured Modus (positive) Detection area (50 bis 500 amu) Curtain Gas (25) Interface Heater (on) Temperature (300°C) Ion spray voltage (5500 V) Declustering-Potential (55 V) Entrance potential (9 V)
MS	
MS (Q-Trap)	Ion source (Turbo Spray) Measured Modus (positive) Detection area (100 bis 500 amu)

	Curtain Gas (10)
	Interface Heater (on)
	Temperature (300°C)
	Ion spray voltage (5500 V)
	Declustering-Potential (110 V)
	Entrance potential (10 V)

Appendix 3. List of solvent and other amount for each device used in this study.

Method	Reagent/solvent	Amount (g/L)/Ratio/MHz	Fracion Nr.	Remarks
Column chromatography	Cyclohexane	2	1–9	negative
		1	10–18	negative
		1	19–27	negative
	Ethylacetat	1	1–9	negative
		1	10–18	negative
		1	19–27	negative
		1.5	28–36	negative
	Methanol	0.3	19–27	negative
		1	28–36	negative
		1	37–45	positive
		1	46–54	negative
		9	55–63	negative
		9	64–72	negative
	H_2O	1	55–63	negative
		5	46–54	negative
		1	73–81	negative
TLC solvents for cysteine derivative	*n*-Butanol	28 ml		
	Water	8 ml		
	Acetic acid	9 ml		

	Formic acid	2 ml
Ninhydrin spray	Ninhydrin	60 g
	Cobalt(II)-nitrate	10 ml
	Acetic acid	2 ml
	Formic acid	0.25 ml
	Collidin	1 ml
	n-Butanol	10 ml

Appendix 3. continued

Buffer for alliinase (after Keusgen, 1999)	KH_2PO_4 (0,026 M)	3.54 g
	Na_2HPO_4 (0,05 M)	7.253 g
	Sucrose (10 %)	100 g
	NaCl (0,08 M)	5 g
	Pyridoxal-5'-phosphat	55 g
	H_2O	*ad* 1L
^{1}H NMR		
2-Ben-PyrrC	CD_3OD	400 MHz
2-Ben-PyrrCSO	D_2O	400 MHz
2-Met-PyrrC	D_2O	400 MHz
2-PyrrC	D_2O	400 MHz
2-ThiophenC	CD_3OD	400 MHz
β-Chloro alanine	D_2O	400 MHz

^{13}C NMR		
2-Ben-PyrrC	CD_3OD	125 MHz
2-Ben-PyrrCSO	D_2O	125 MHz
2-Met-PyrrC	D_2O	125 MHz
2-PyrrC	D_2O	125 MHz
2-eC	CD_3OD	125 MHz
β-Chloro alanine	D_2O	125 MHz
HPLC	EC 250/4 Nucleodur 100-5 C18ec	
HPLC-MS	EC 250/2 Nucleodur 100-5 C18ec	

Appendix 4. Synthesis of S-(N-benzylpyrrol-2-yl)cysteine using analytical HPLC.

Time (min)	%Wasse	%Wasse	%Metha	%Metha	Flow (ml/min)	Event 1	Event 2	Event 3	Event 4
0,0	60,0	0,0	0,0	40,0	0,700	Pulse			
30,0	60,0	0,0	0,0	40,0	0,700				
50,0	5,0	0,0	0,0	95,0	0,700				
55,0	5,0	0,0	0,0	95,0	0,700				

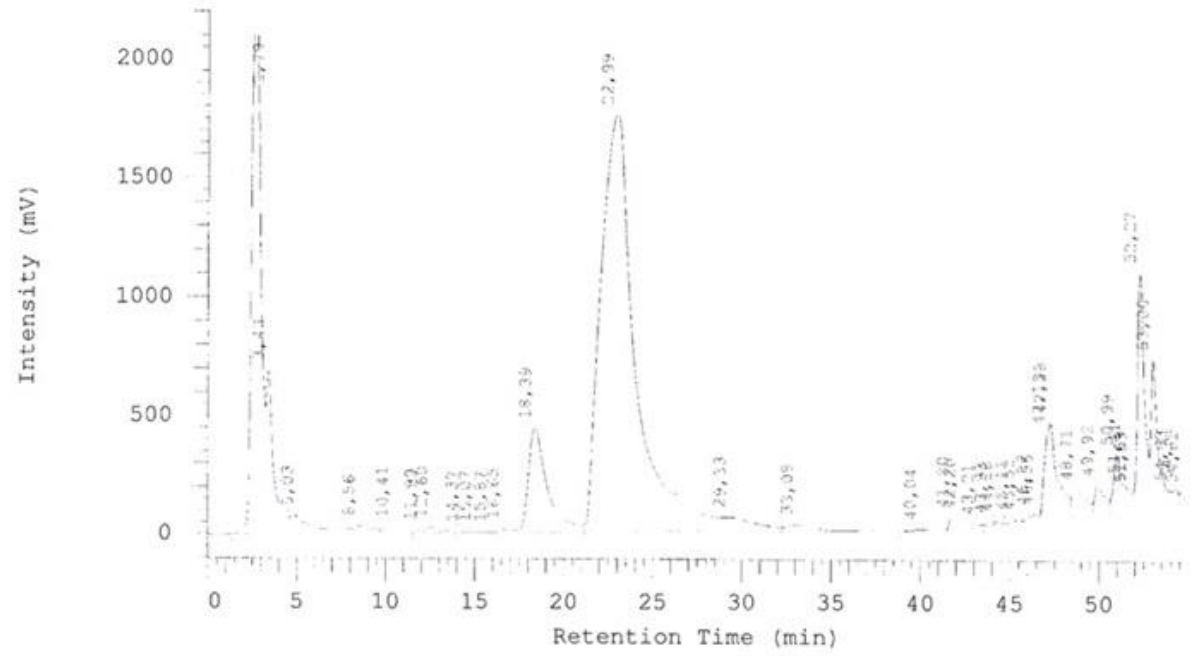
Appendix 5. ESI-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide before preparative-HPLC.

Time (min)	%Aceto	%Wasse	%Metha	%Wasse	Flow (ml/min)	Event 1	Event 2	Event 3	Event 4
0,0	0,0	0,0	37,0	63,0	0,700				
15,0	0,0	0,0	37,0	63,0	0,700				
20,0	0,0	0,0	95,0	5,0	0,700				
30,0	0,0	0,0	95,0	5,0	0,700				

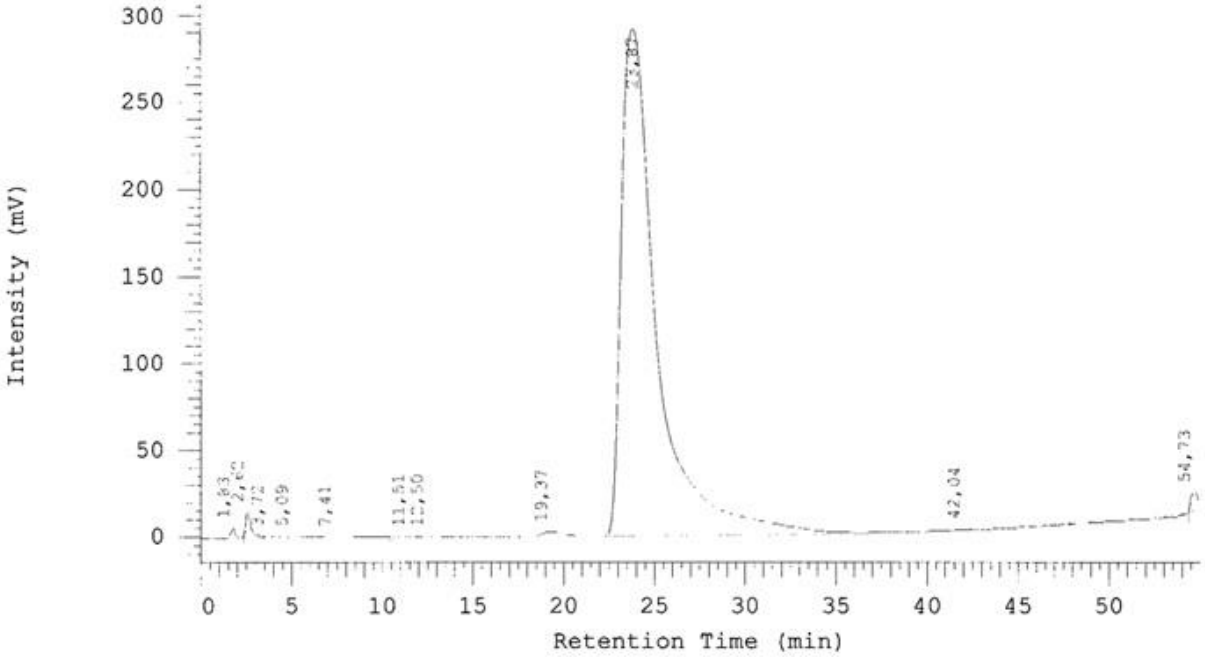
Appendix 6. Analytical HPLC method condition for enzymatic bioactivity test.

Time	**Water (%)**	**Methanol (%)**	**Flow rate (ml/min)**	**Pressure (mbar)**
0	95	5	1	0–400
2	95	5	1	0–400
20	30	70	1	0–400
55	5	95	1	0–400
60	5	95	1	0–400

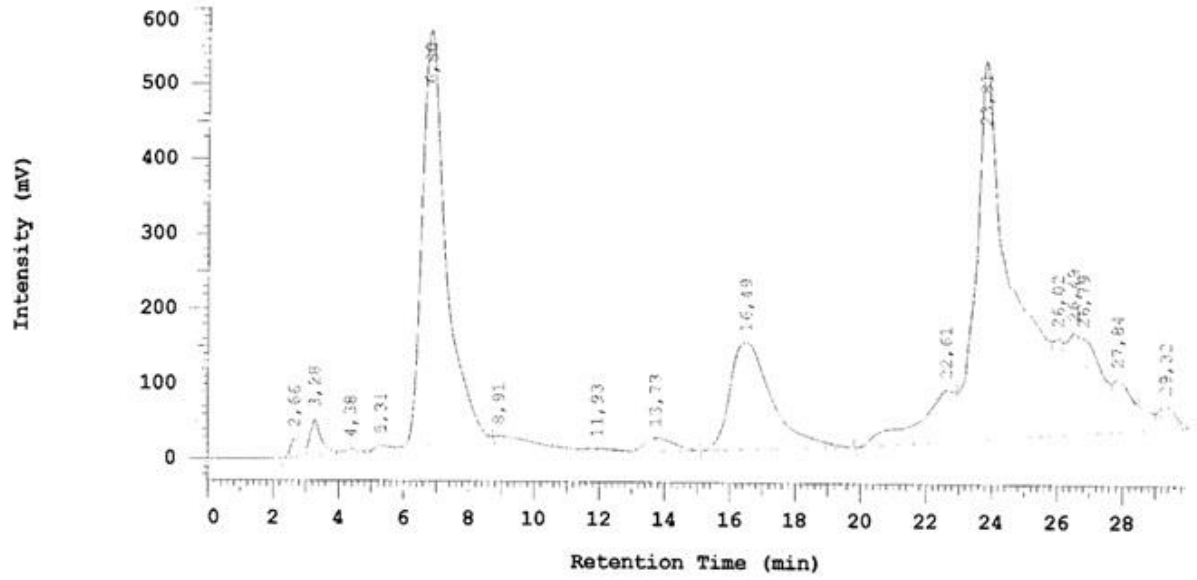
Appendix 7. Chromatogram of S-(N-benzylpyrrol-2-yl)cysteine using analytical HPLC.



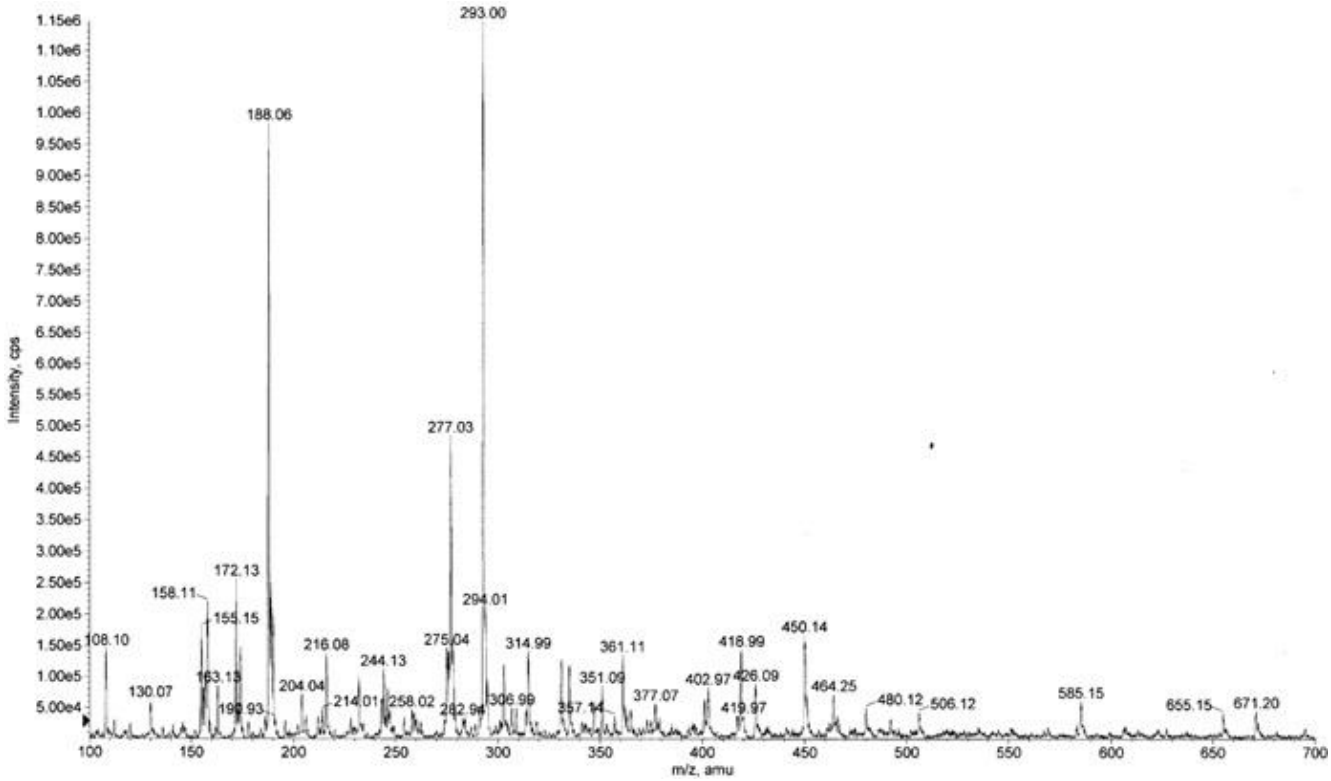
Appendix 8. Chromatogram of S-(N-benzylpyrrol-2-yl)cysteine non-oxide after preparative HPLC.



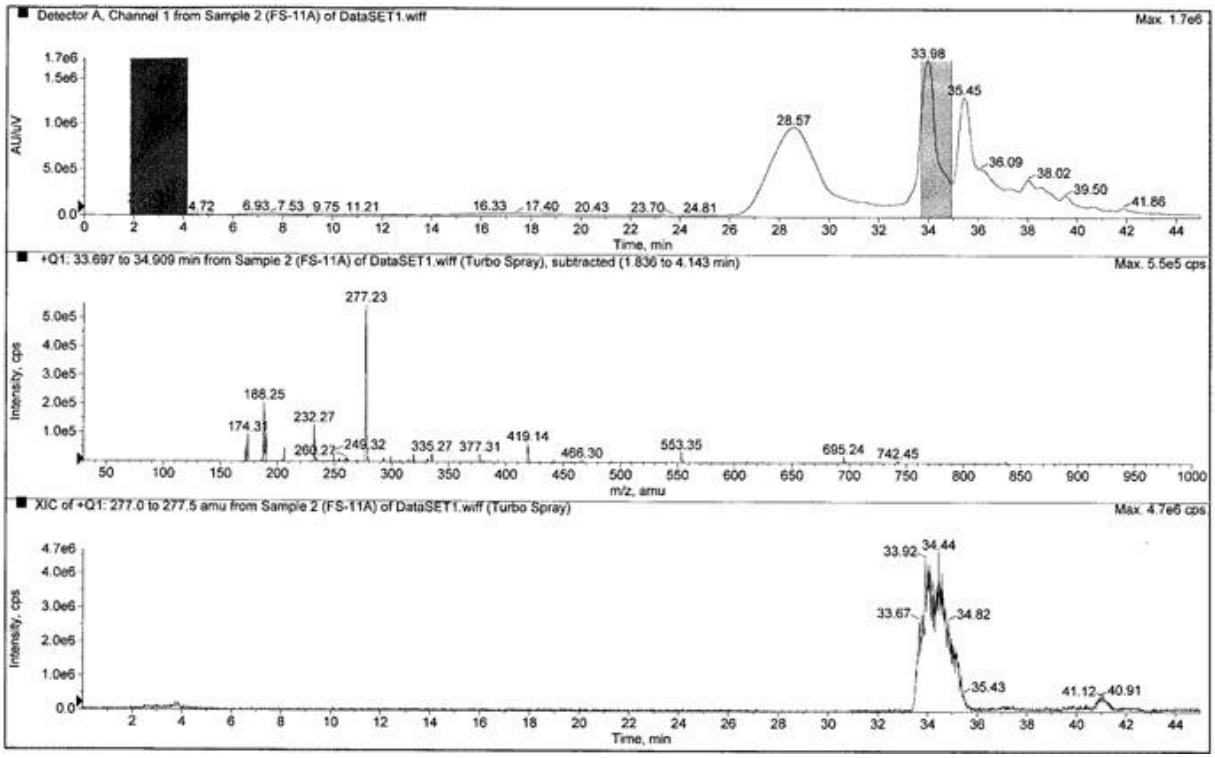
Appendix 9. HPLC chromatogram of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide before preparative-HPLC.



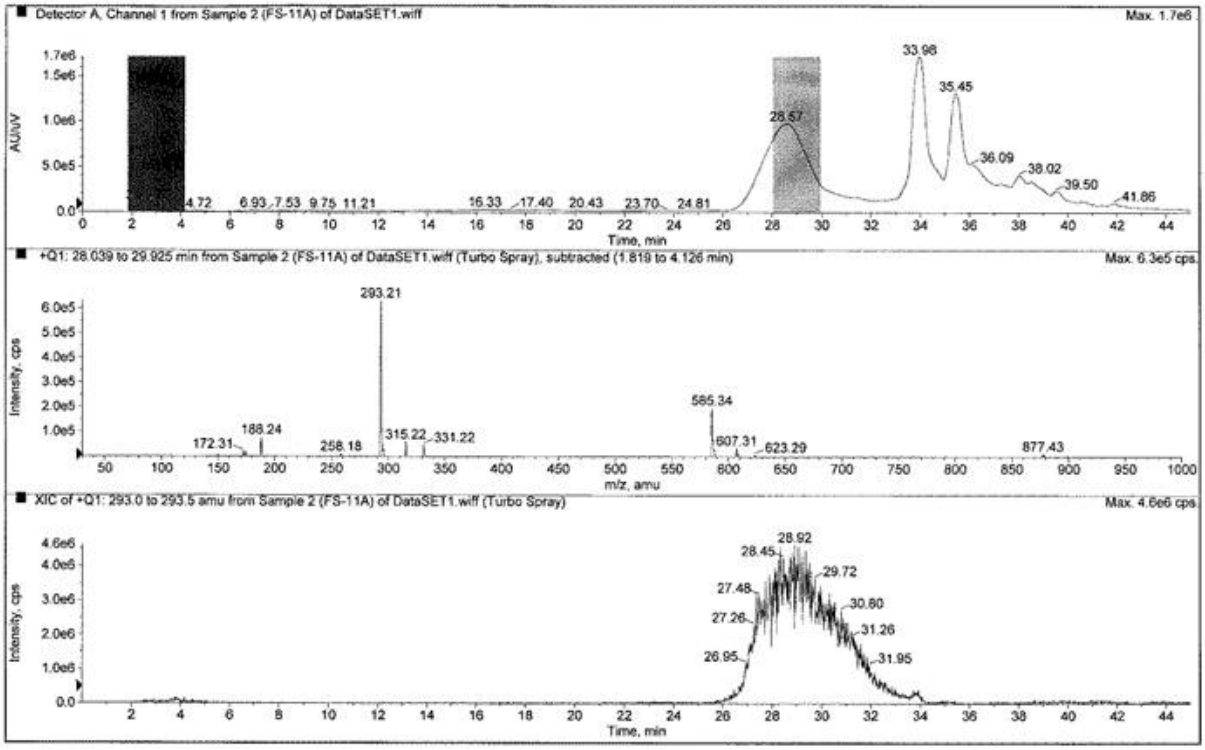
Appendix 10. ESI-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide before preparative-HPLC.



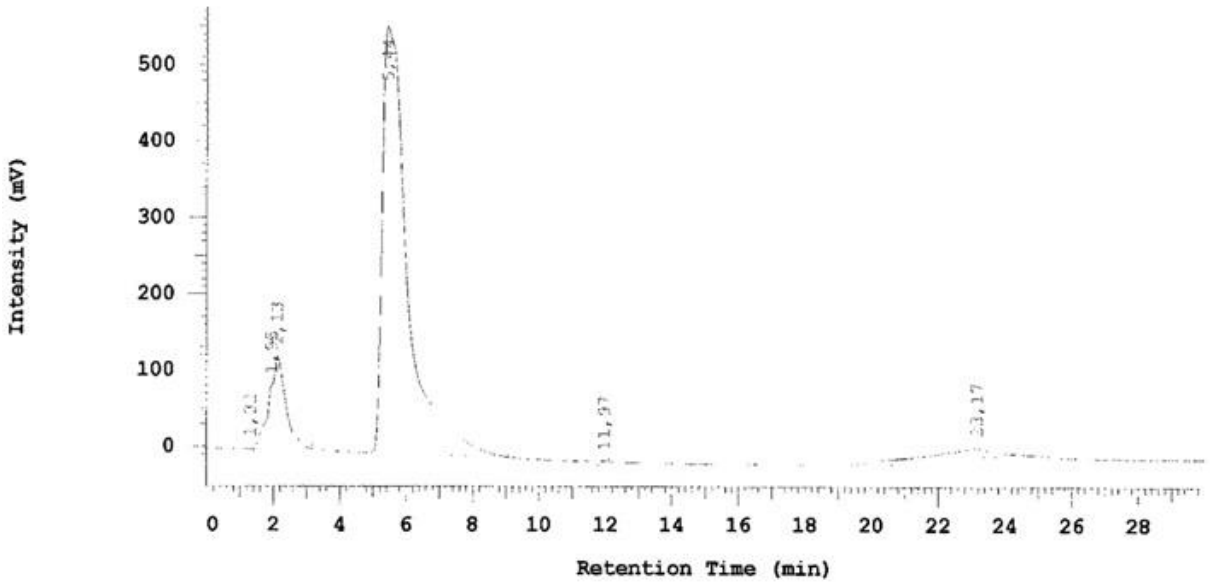
Appendix 11. HPLC-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide.



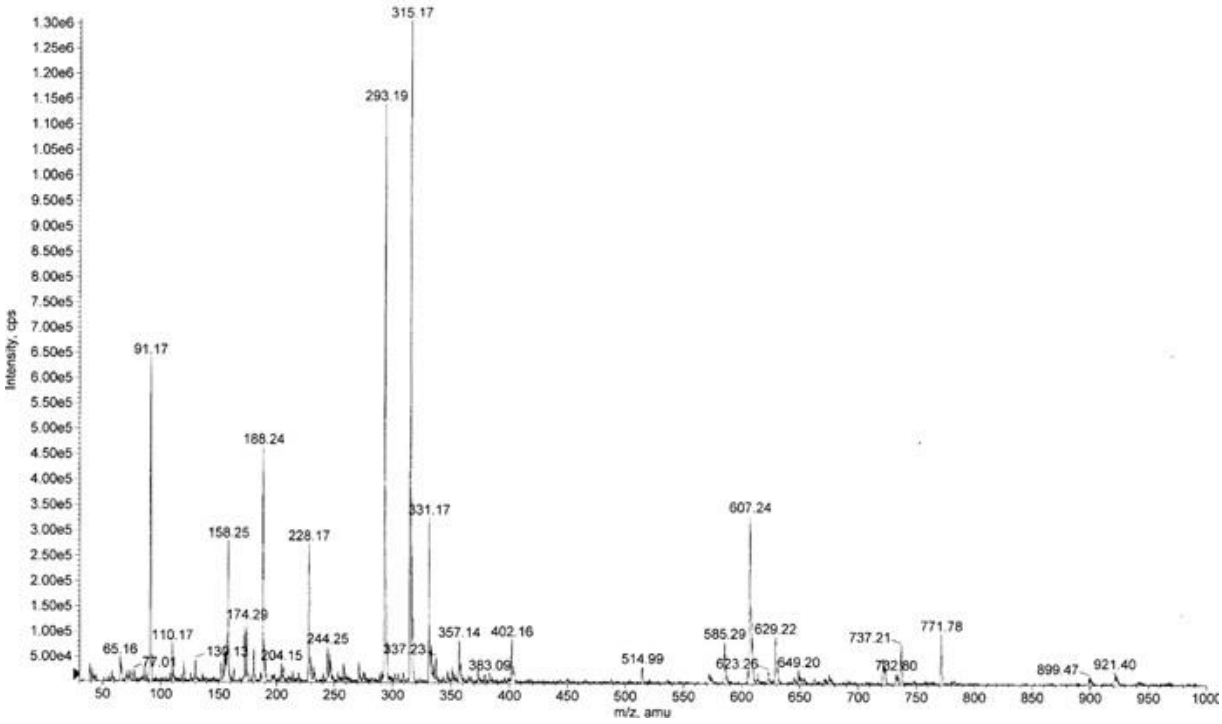
Appendix 12. HPLC-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine.



Appendix 13. HPLC analysis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide after preparative-HPLC.



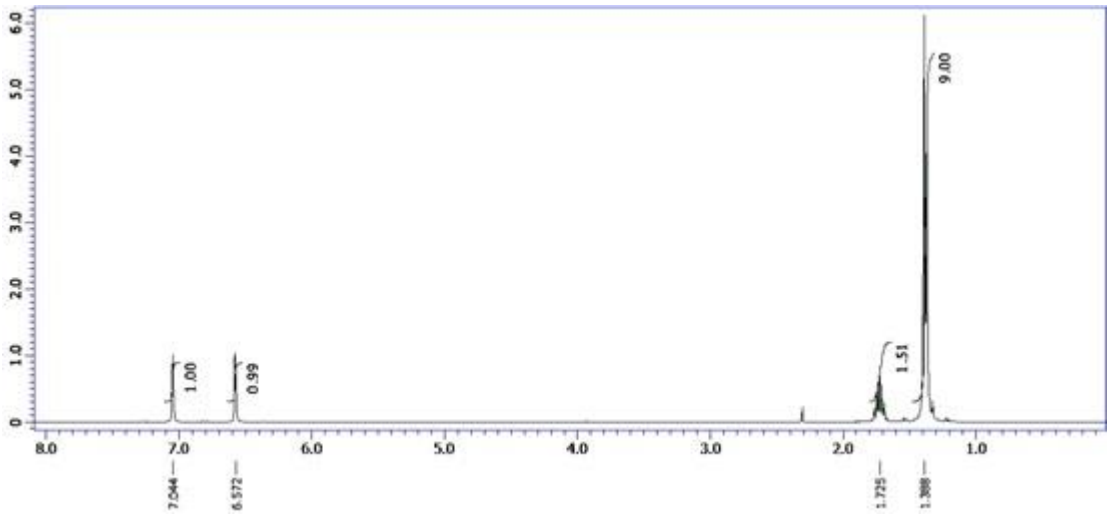
Appendix 14. ESI-MS analysis of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide after preparative-HPLC.



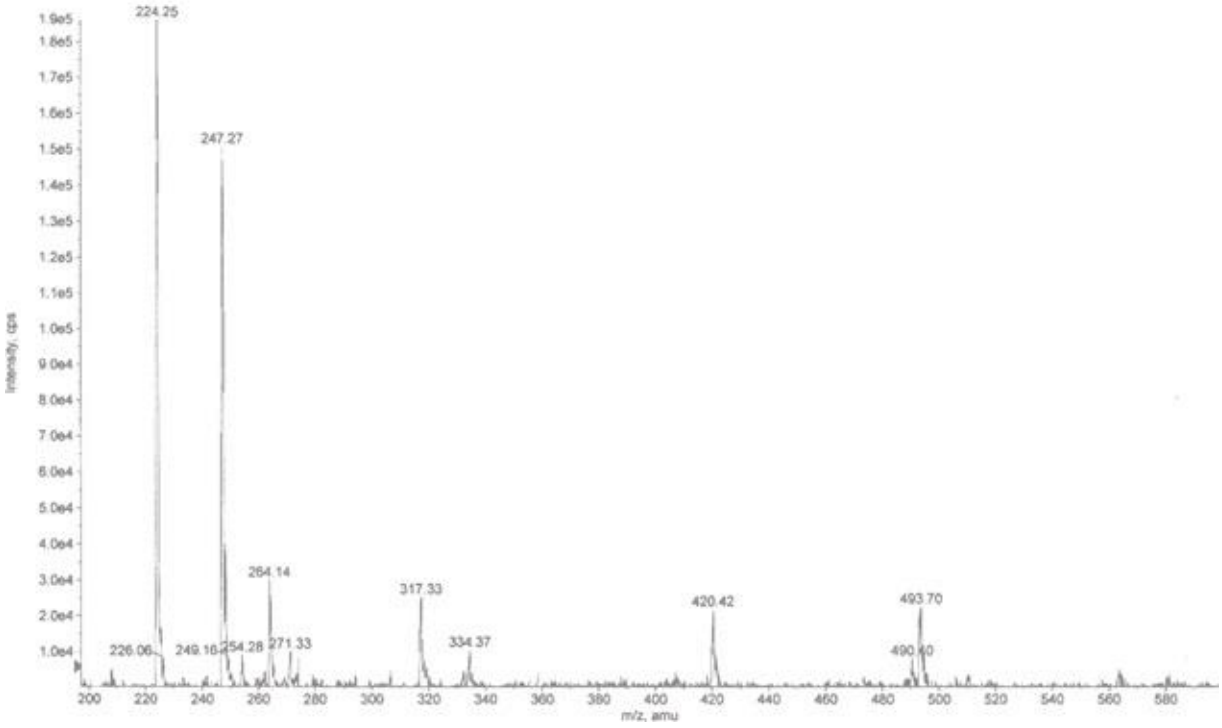
Appendix 15. Measurement of TLC spot quantification of S-(2-thienyl)cysteine-S-oxide.

Number	µg	Volume	Height	Area	Percent %
non-ox	100	11130	20	1935	3,7
sulfoxid	1269,461	141291	67	3375	47,3
sulfonic acid	1312,489	146080	79	3285	48,9

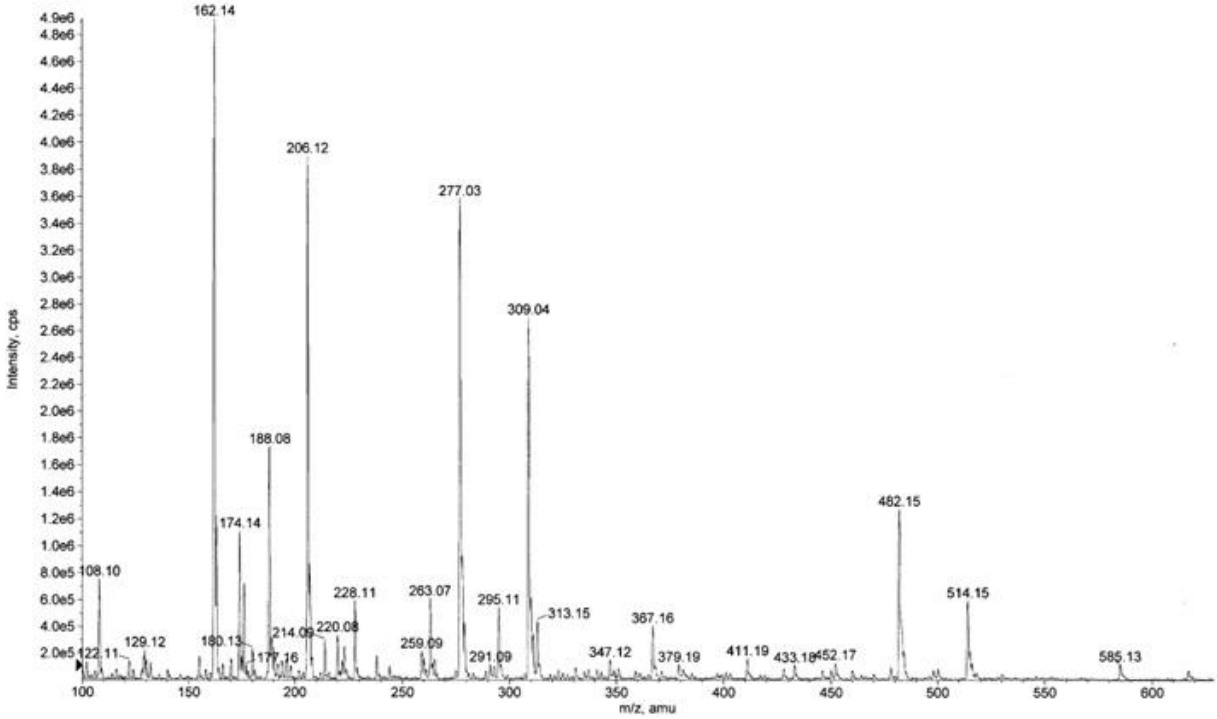
Appendix 16. 1H-NMR spectrum of 1-(triisopropylsilyl)pyrrole



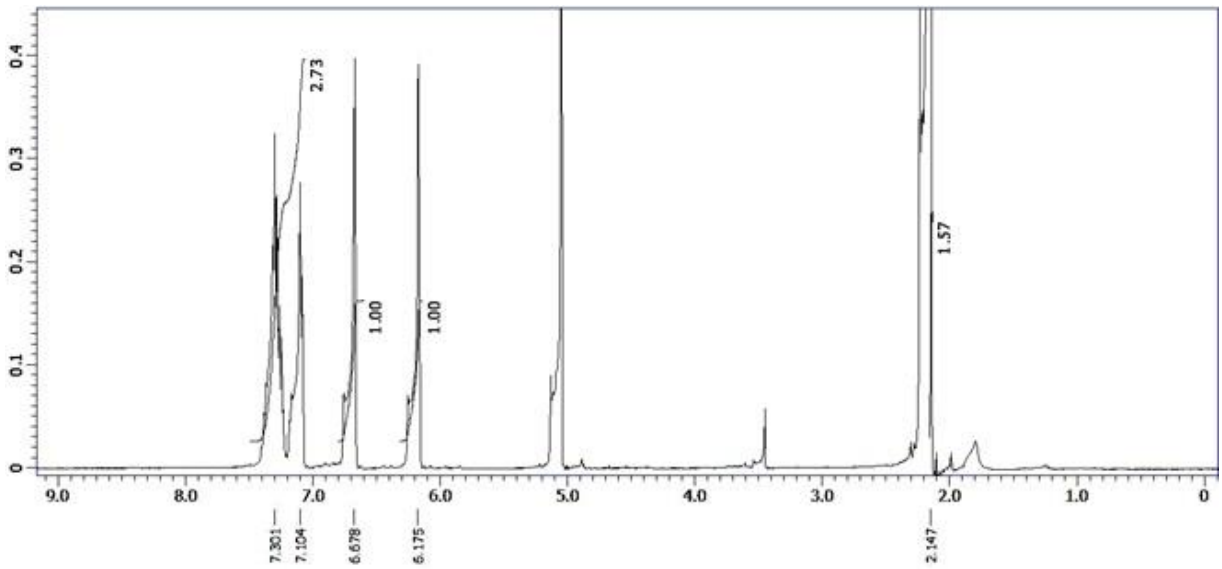
Appendix 17. ESI-MS analysis of 1-(triisopropylsilyl)pyrrole



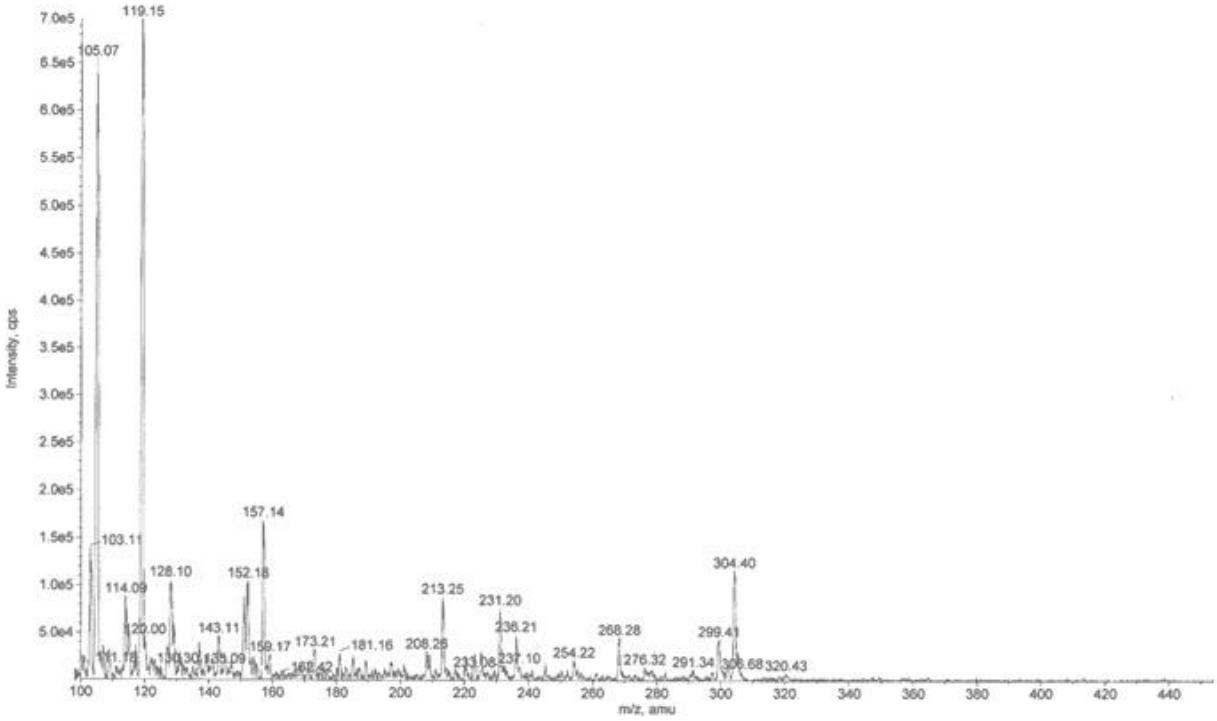
Appendix 18. ESI-MS analysis of hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide with pd/C and hydrogen.



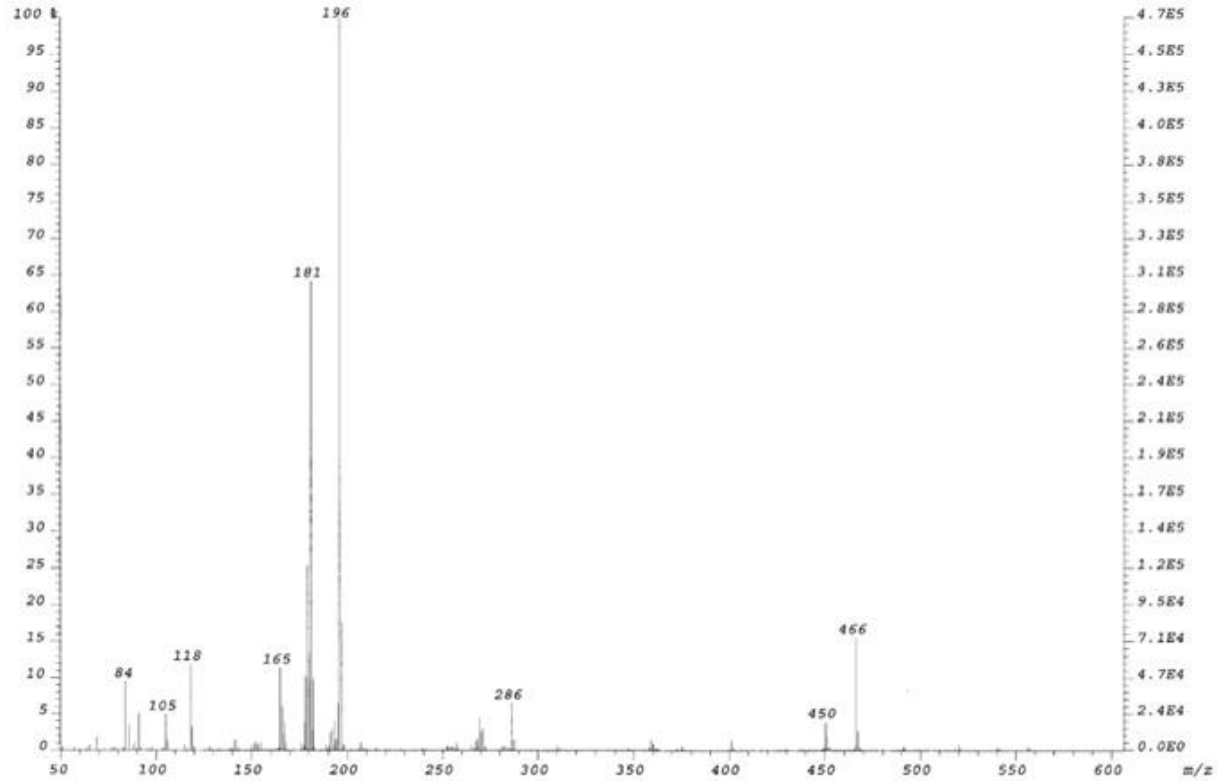
Appendix 19. ^{1}H-NMR spectrum of hydrogenated N-benzyl pyrrole.



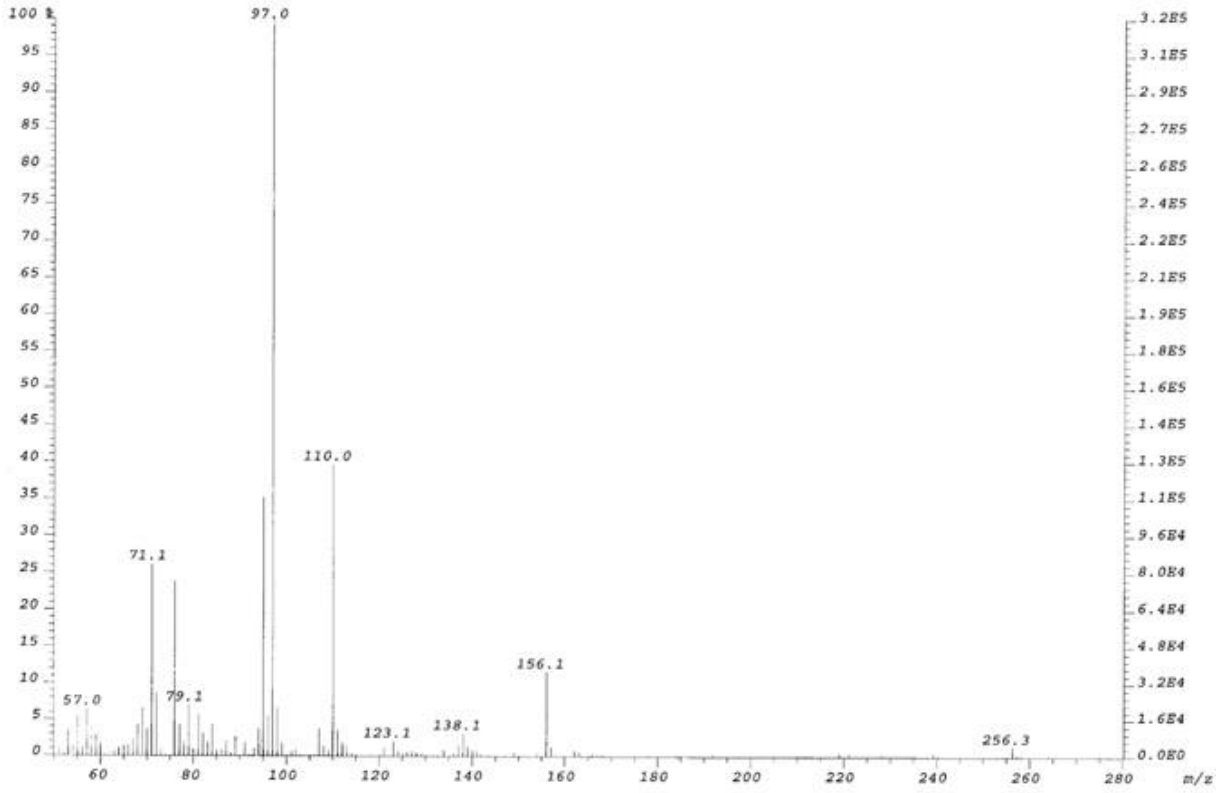
Appendix 20. ESI-MS analysis of S-(N-Bocpyrrol-2-yl)cysteine.



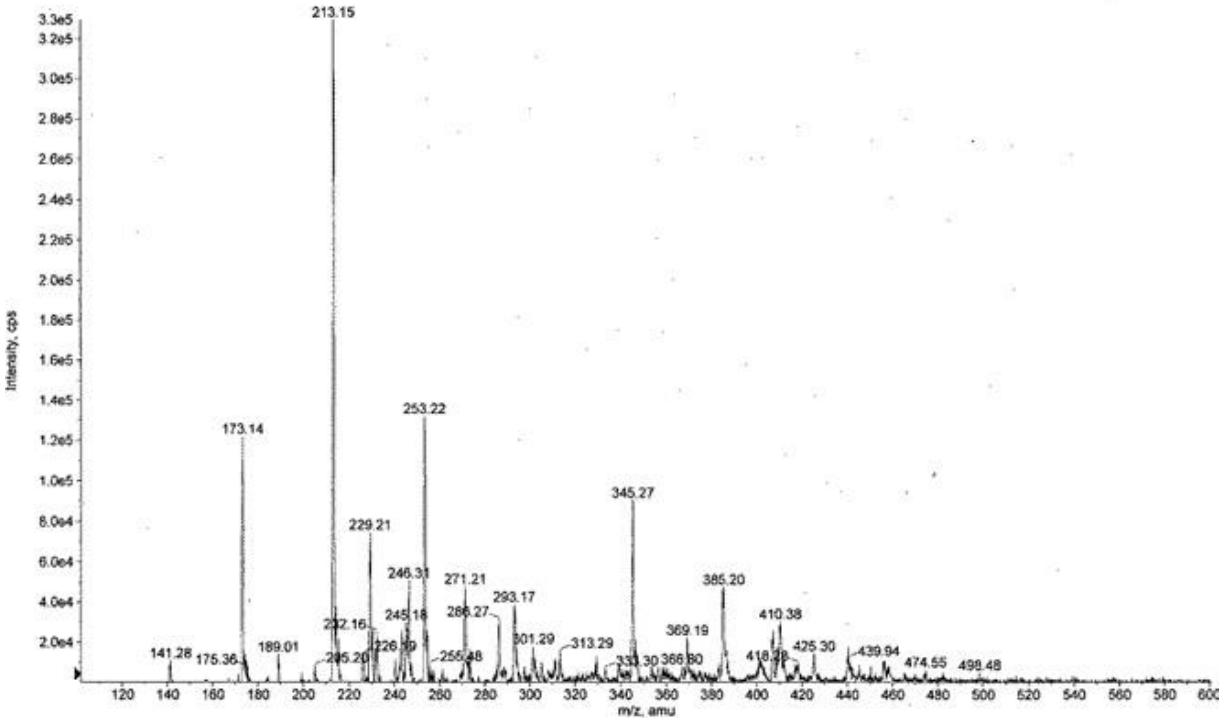
Appendix 21. ESI-MS analysis of Cbz-serine benzylester-O-trifilate.



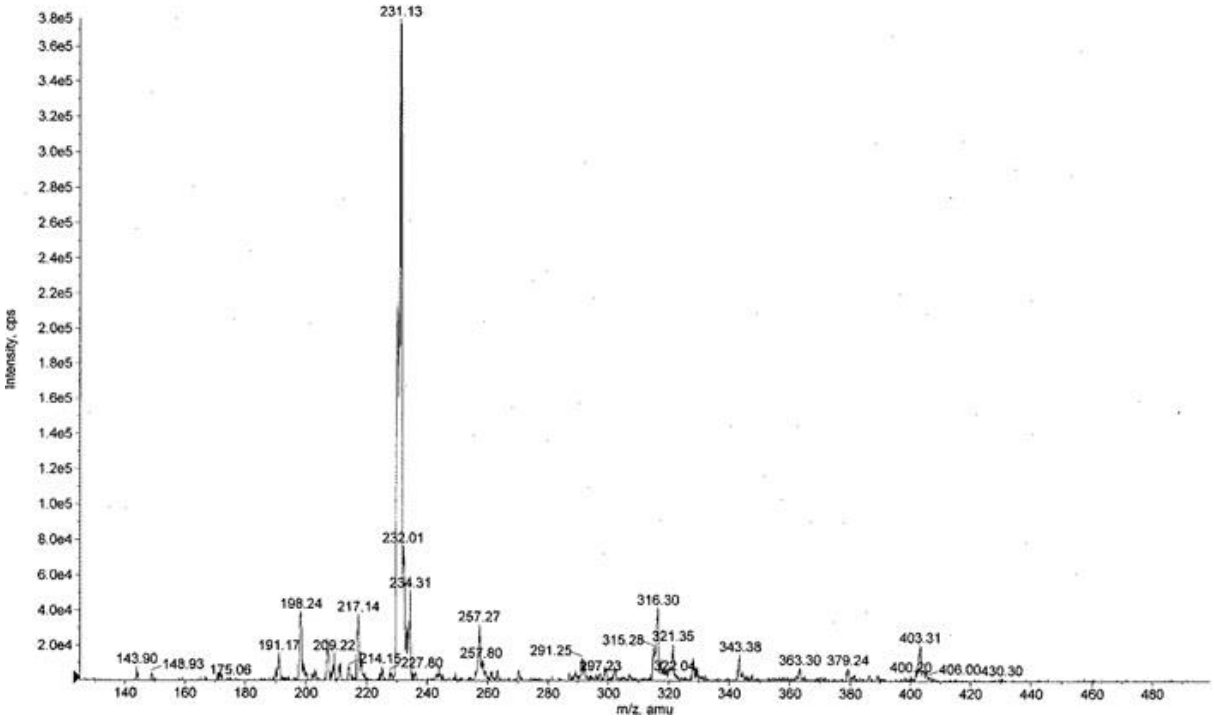
Appendix 22. ESI-MS analysis of lithium furan-2-thiolate.



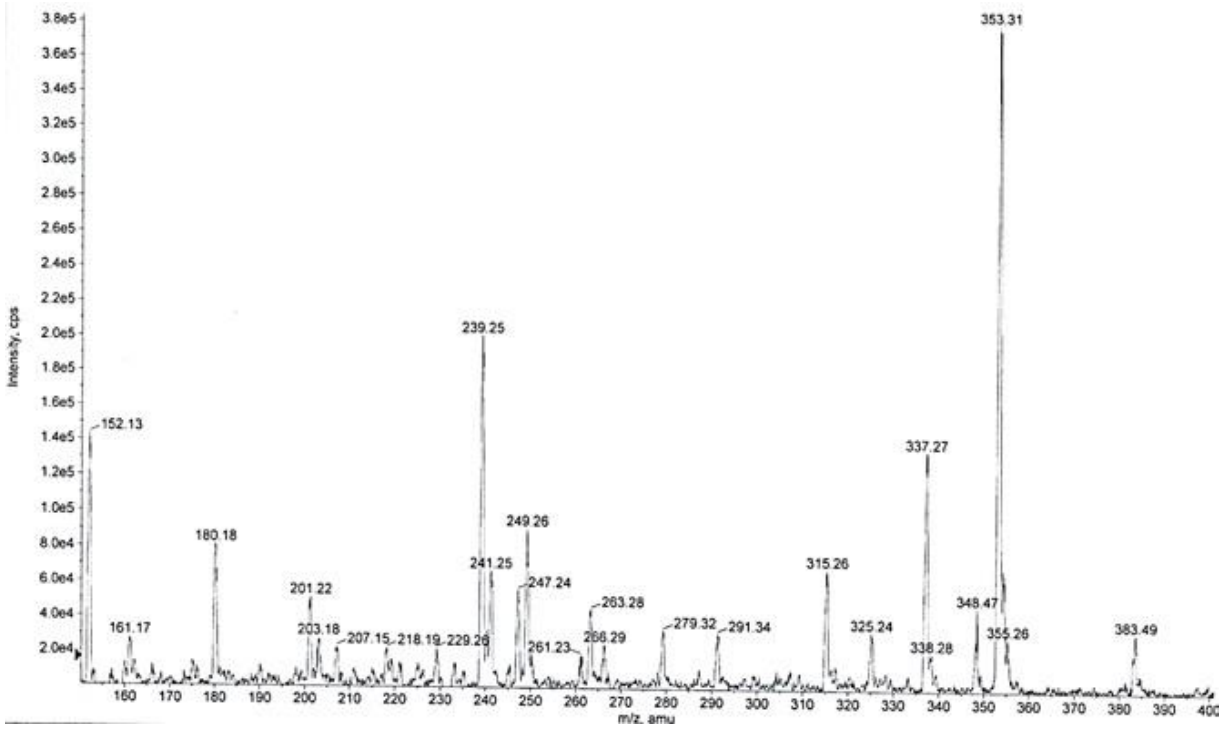
Appendix 23. ESI-MS analysis of S-(2-pyrrolyl)cysteine (+Q).



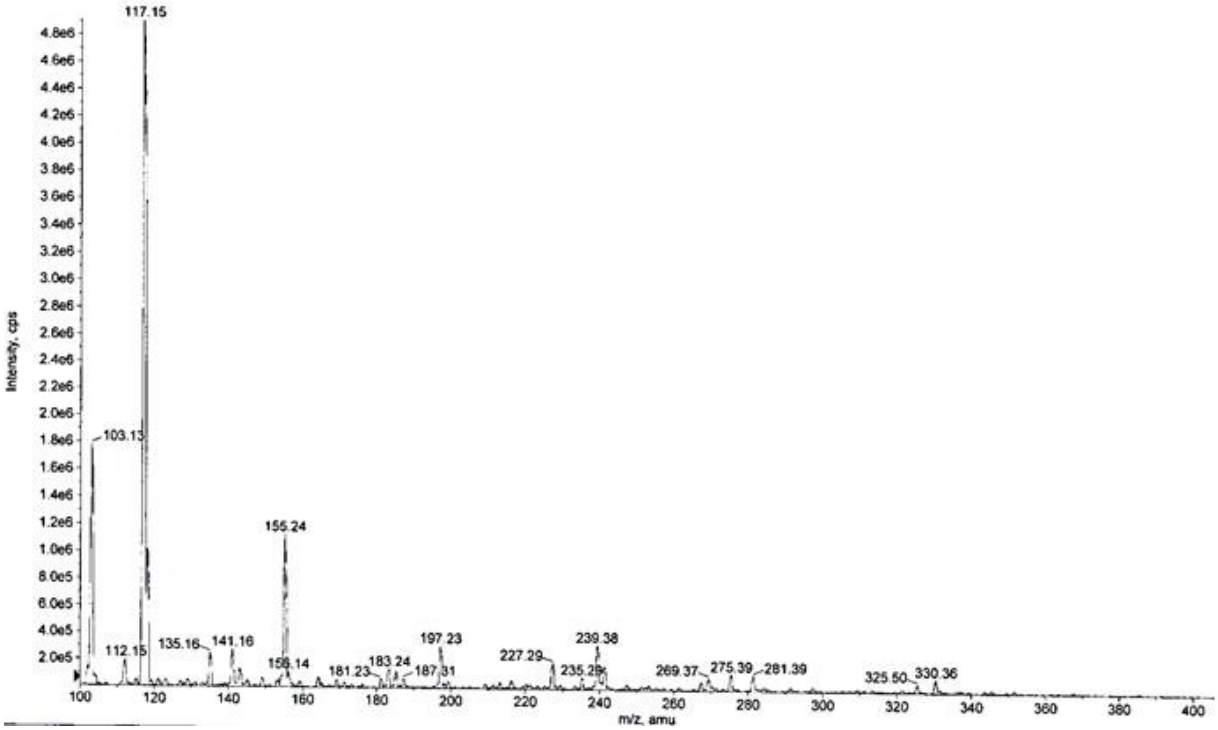
Appendix 24. ESI-MS analysis of S-(2-pyrrolyl)cysteine (-Q).



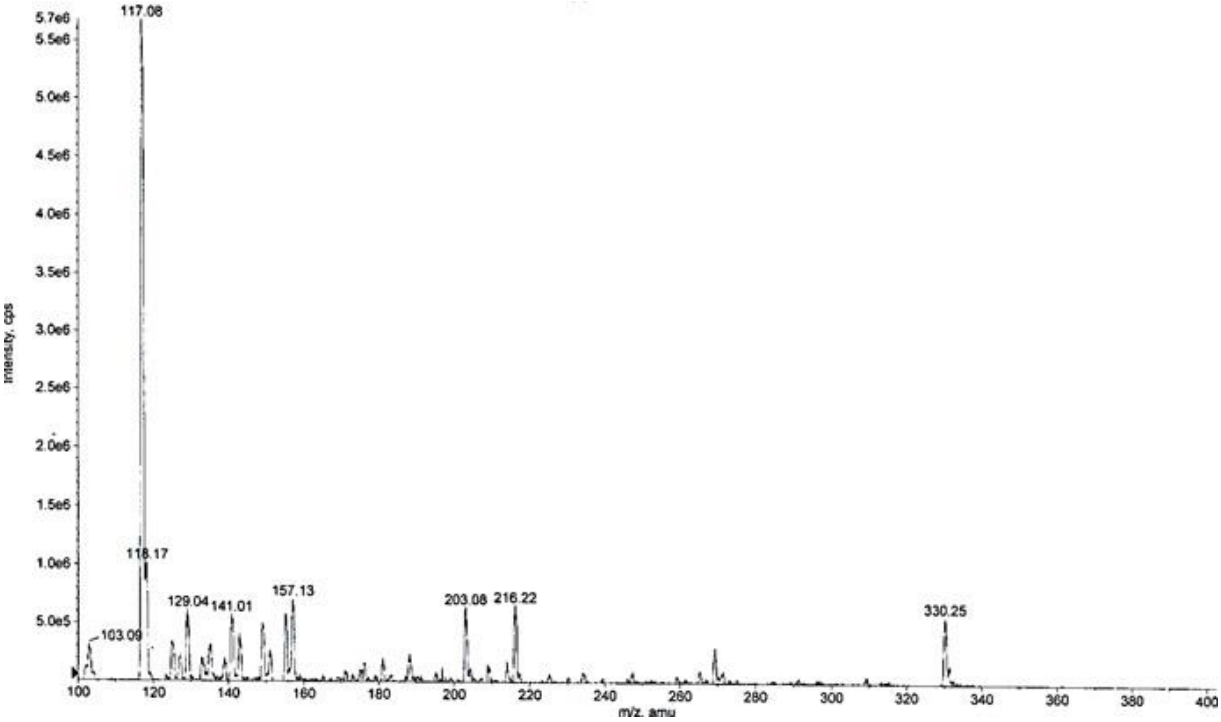
Appendix 25. ESI-MS analysis of trifluoromethylsulfonyl-serine with trimethylamine



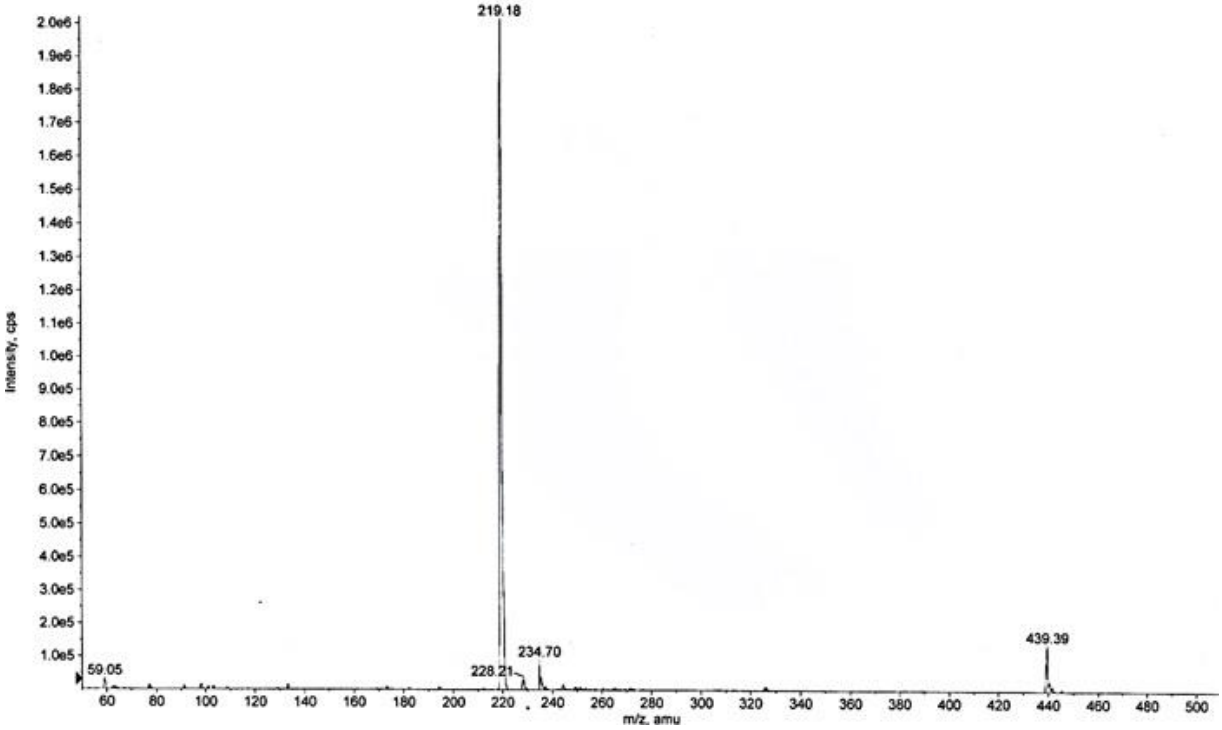
Appendix 26. ESI-MS analysis of S-(3-thienyl)cysteine



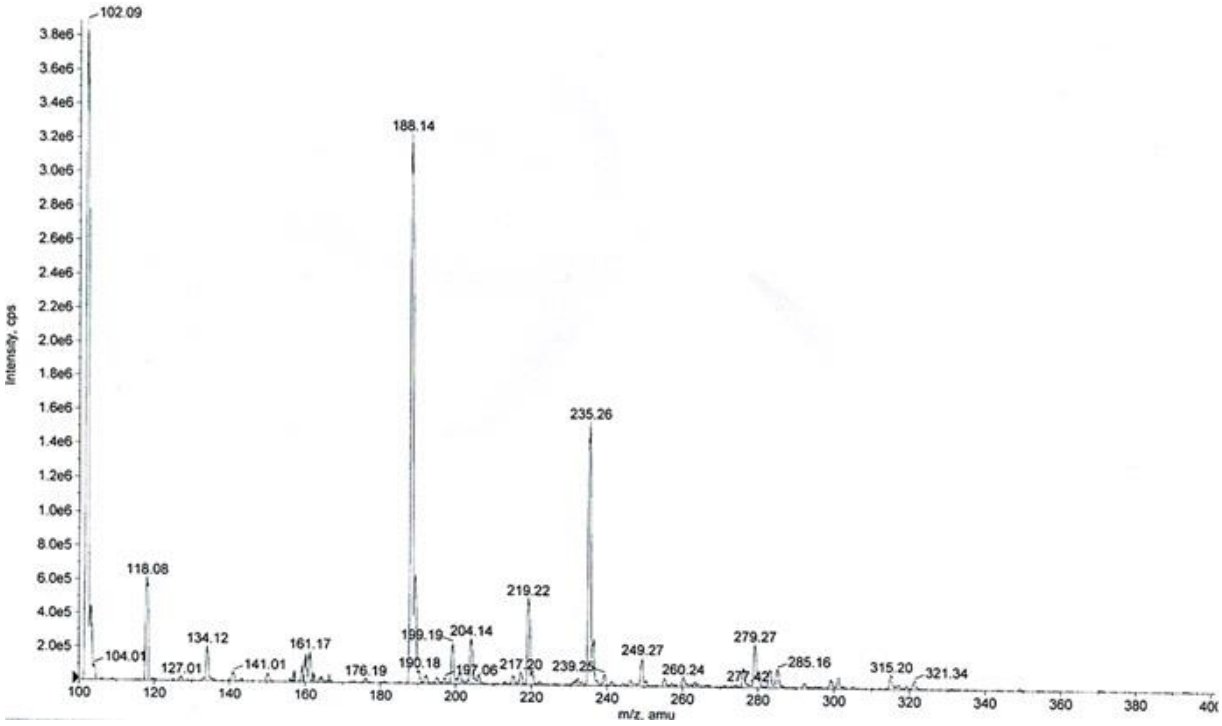
Appendix 27. ESI-MS analysis of Boc-S-(N-methylpyrrol-2-yl)cysteine (+Q).



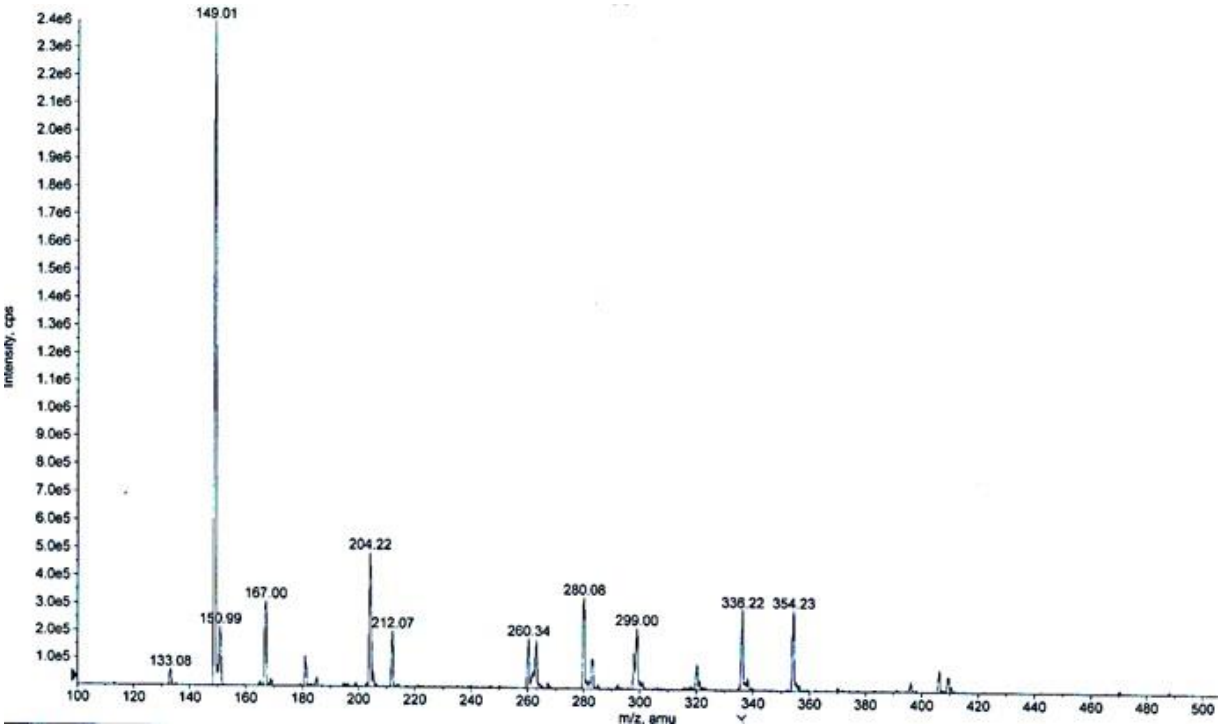
Appendix 28. ESI-MS analysis of Boc-S-(N-methylpyrrol-2-yl)cysteine (-Q1)



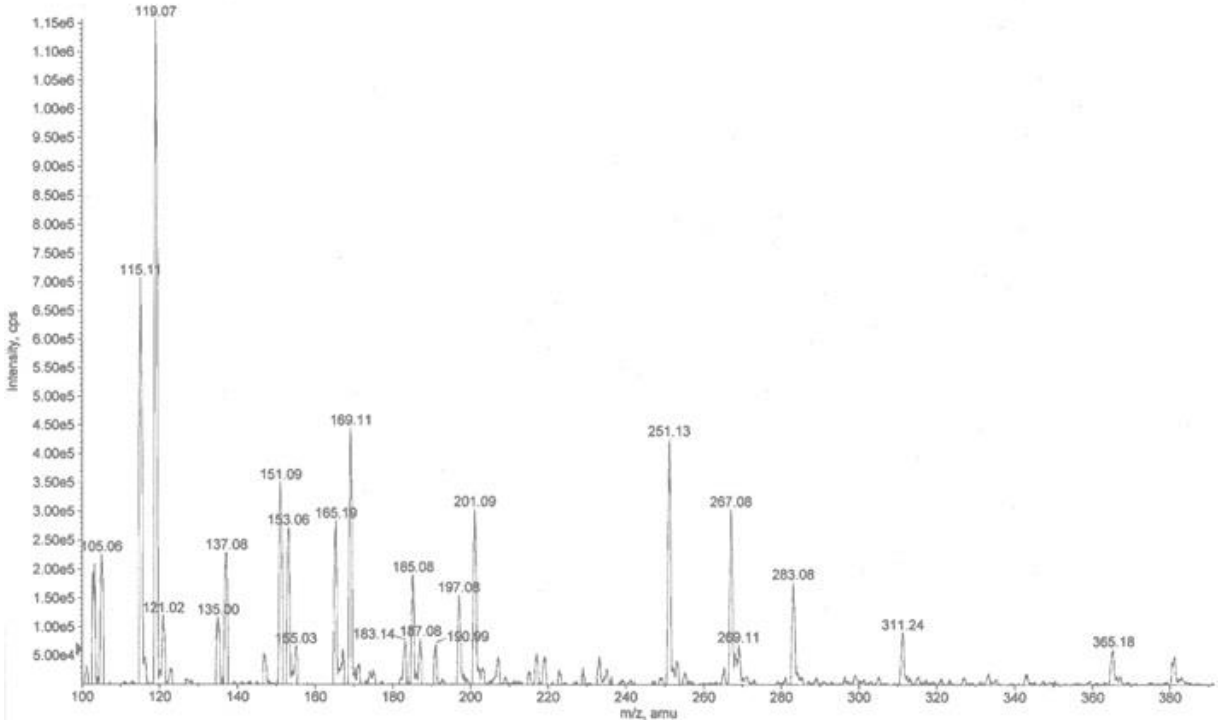
Appendix 29. ESI-MS analysis of 3-bromo N-methylpyrrole



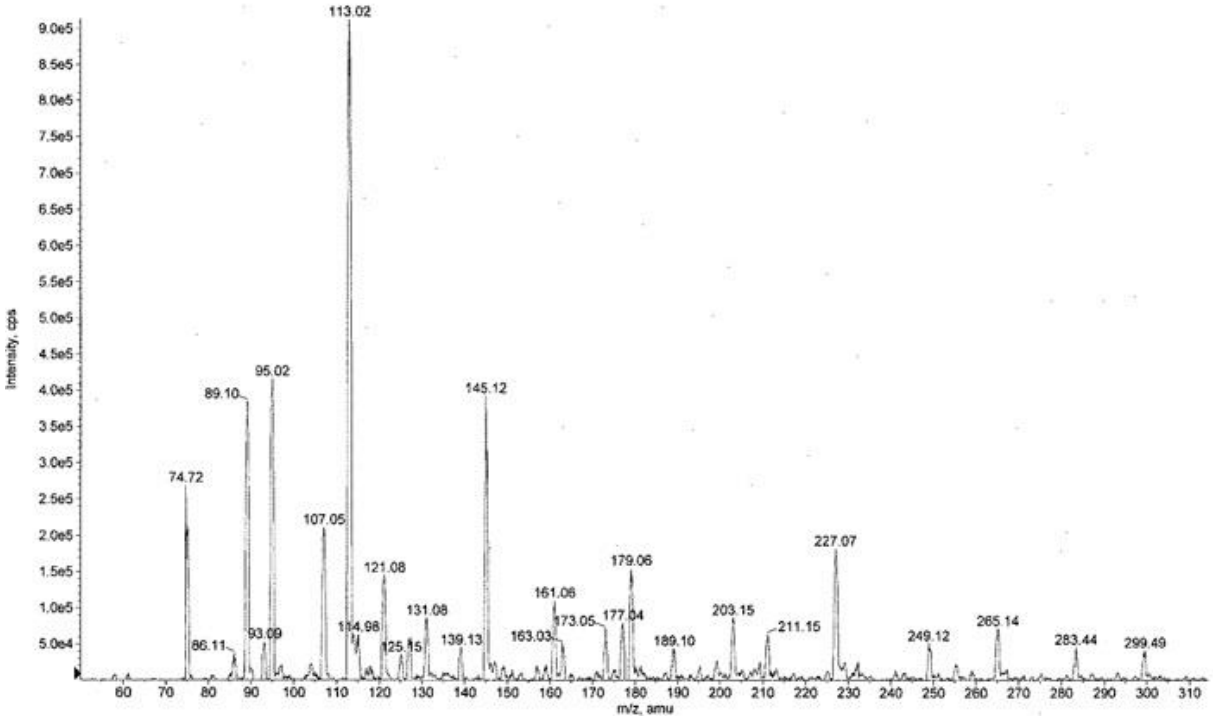
Appendix 30. ESI-MS analysis of N-(tert-butoxycarbonyl)-O-trifluoromethylsulfonyl-serine with 2,6-lutidine



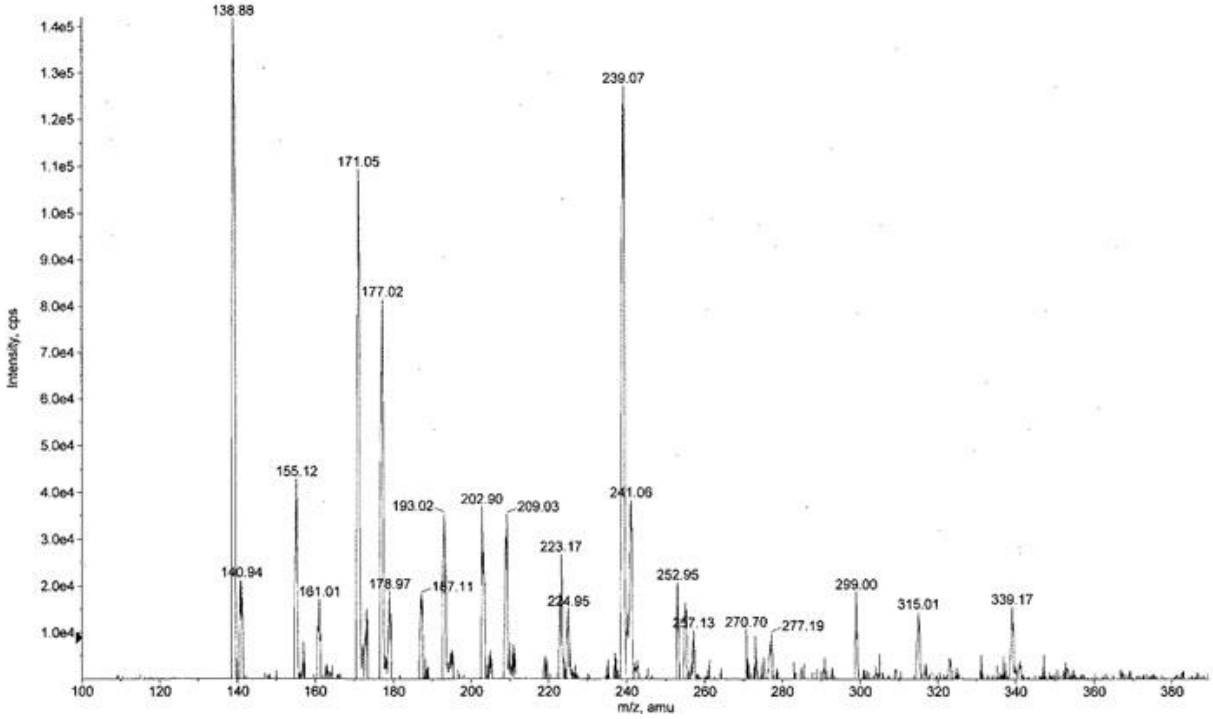
Appendix 31. ESI-MS analysis of S-(2-imidazolyl)cysteine (+Q).



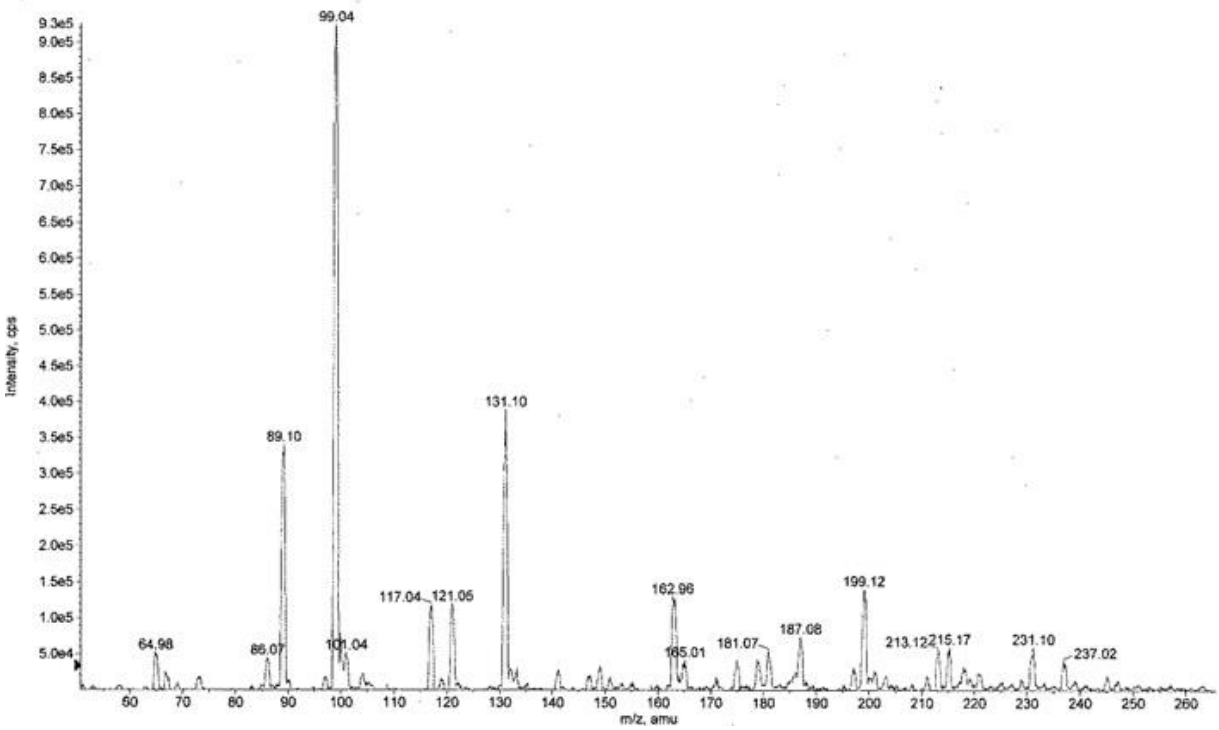
Appendix 32. ESI-MS analysis of S-(2-imidazolyl)cysteine (-Q)



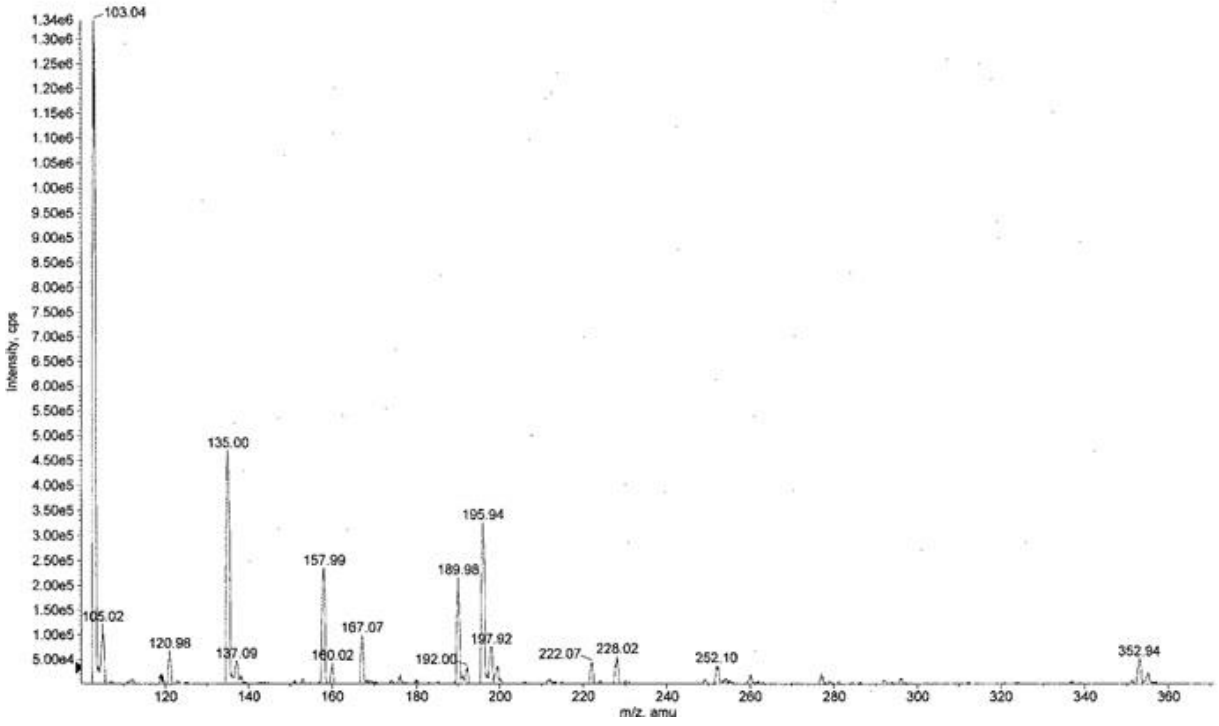
Appendix 33. ESI-MS analysis of S-(N-methylimidazol-2-yl)cysteine (+Q).



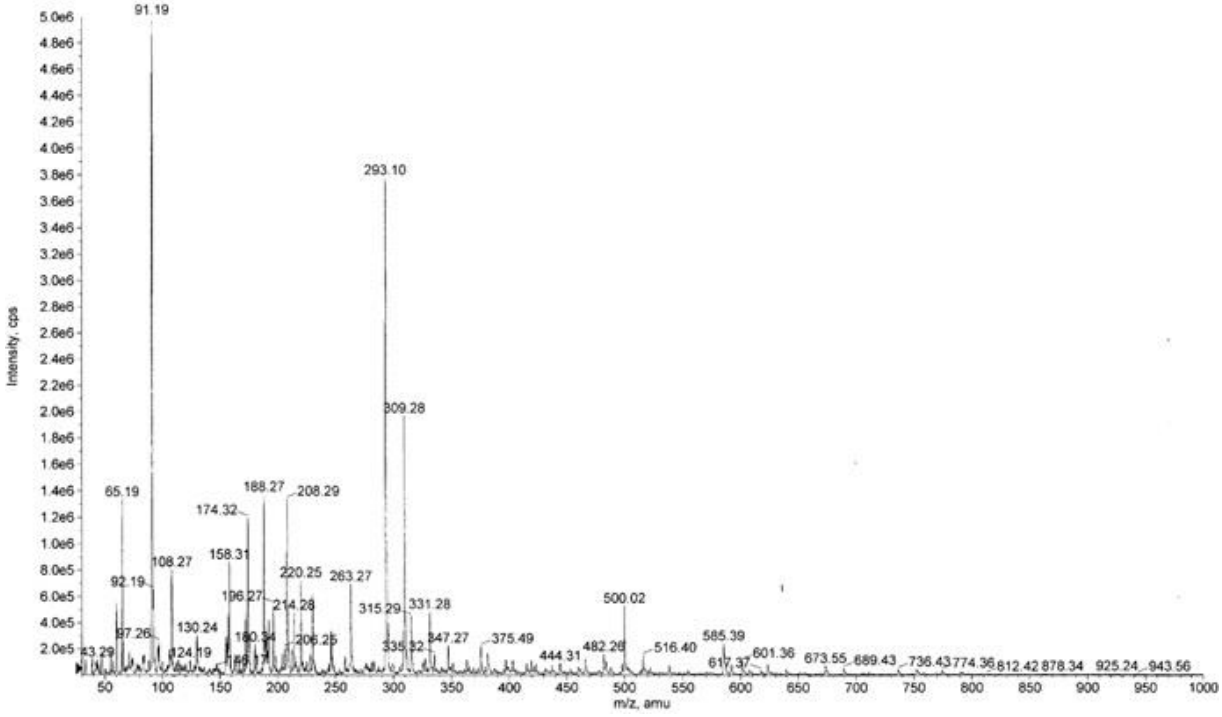
Appendix 34. ESI-MS analysis of S-(N-methylimidazol-2-yl)cysteine (-Q).



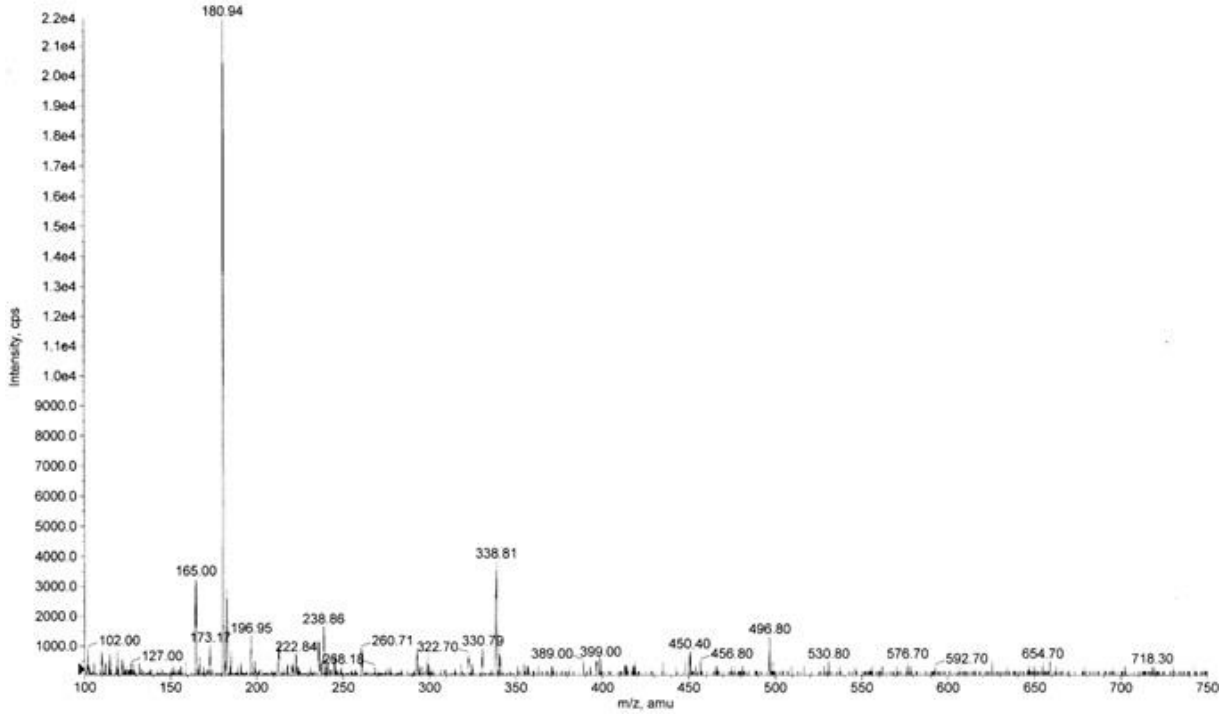
Appendix 35. ESI-MS analysis of S-(2-thiazolinyl)cysteine (+Q).



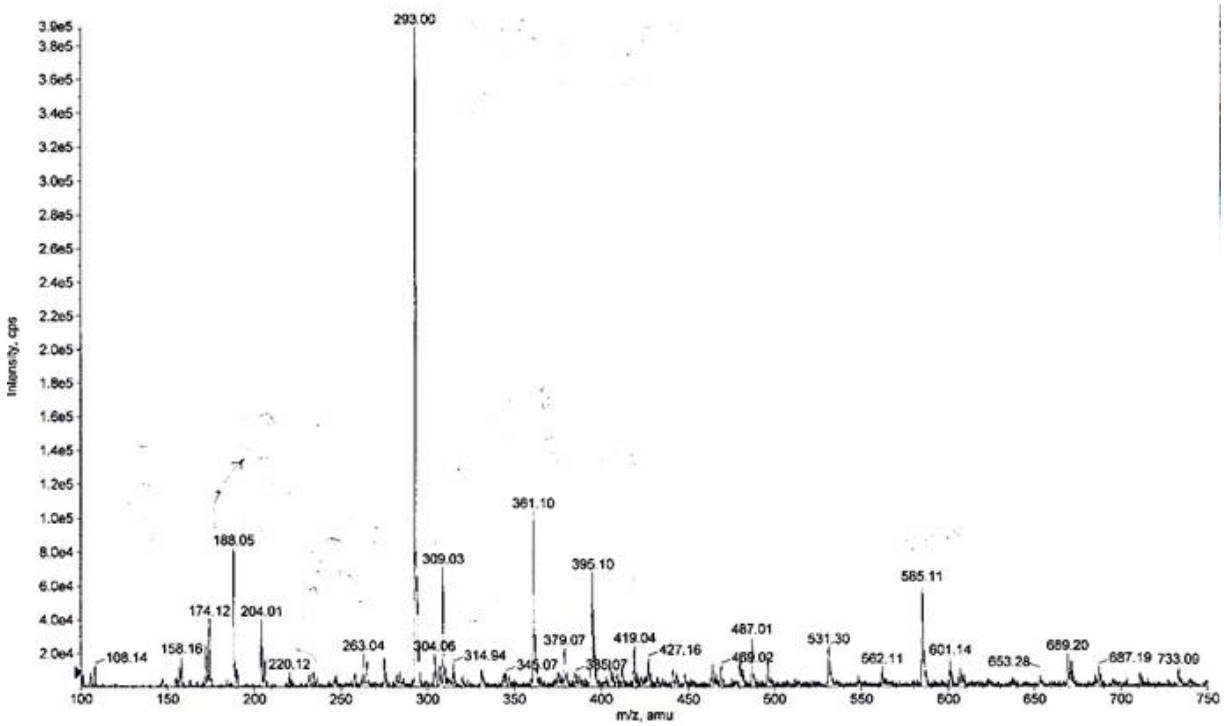
Appendix 36. ESI-MS analysis of oxidation of S-(N-benzylpyrrol-2-yl)cysteine with H_2O_2 in acetic acid.



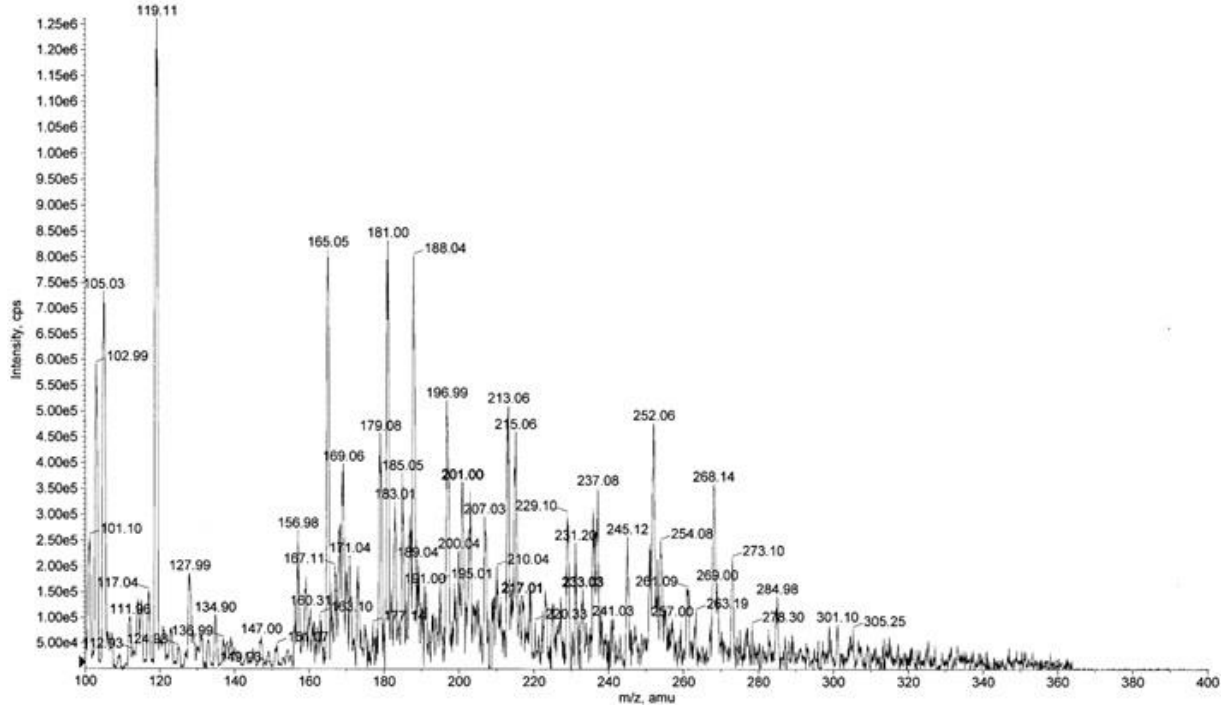
Appendix 37. ESI-MS analysis of oxidation of S-(N-benzylpyrrol-2-yl)cysteine with $FeCl_3$/ H_5IO_6.



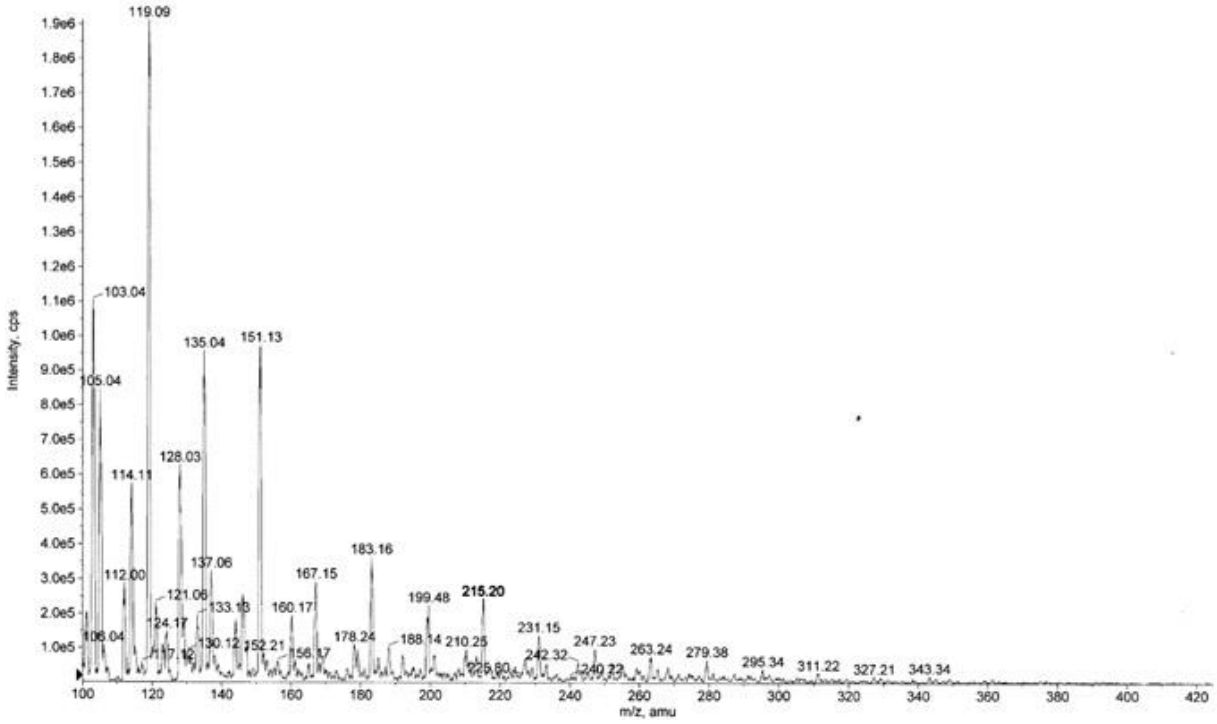
Appendix 38. ESI-MS analysis of oxidation with MMPP (magnesium monoperoxyphthalate).



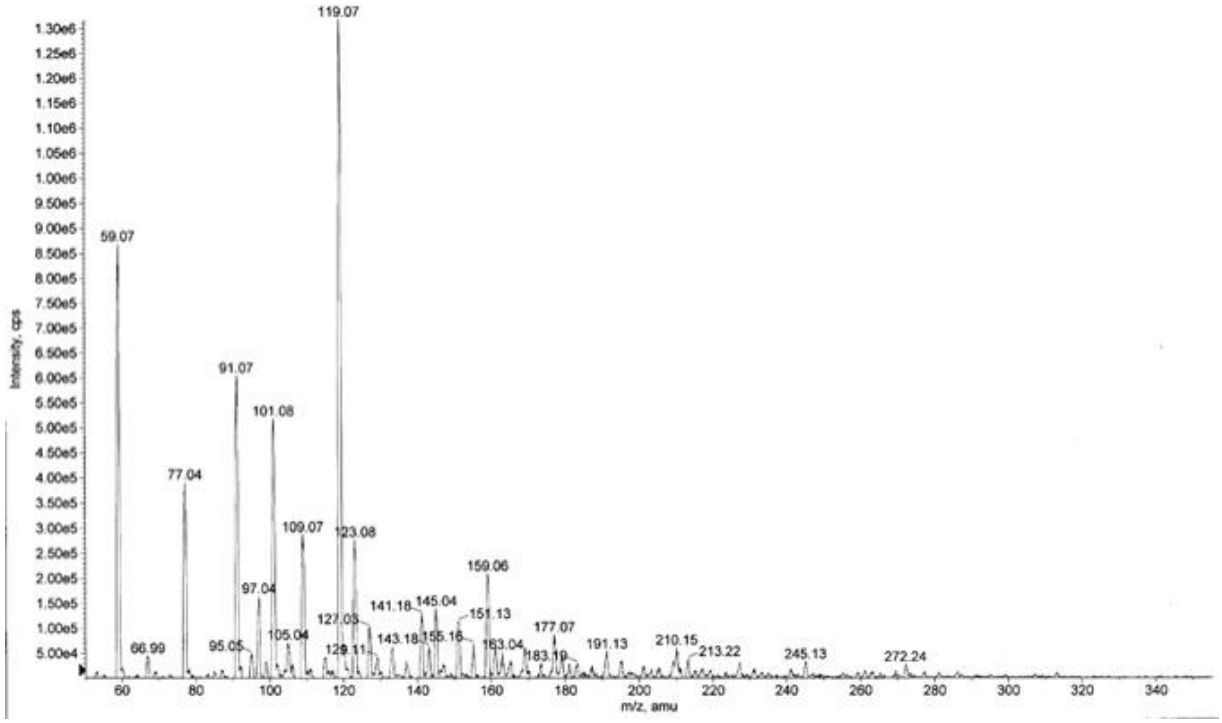
Appendix 39. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with H_2O_2 in water.



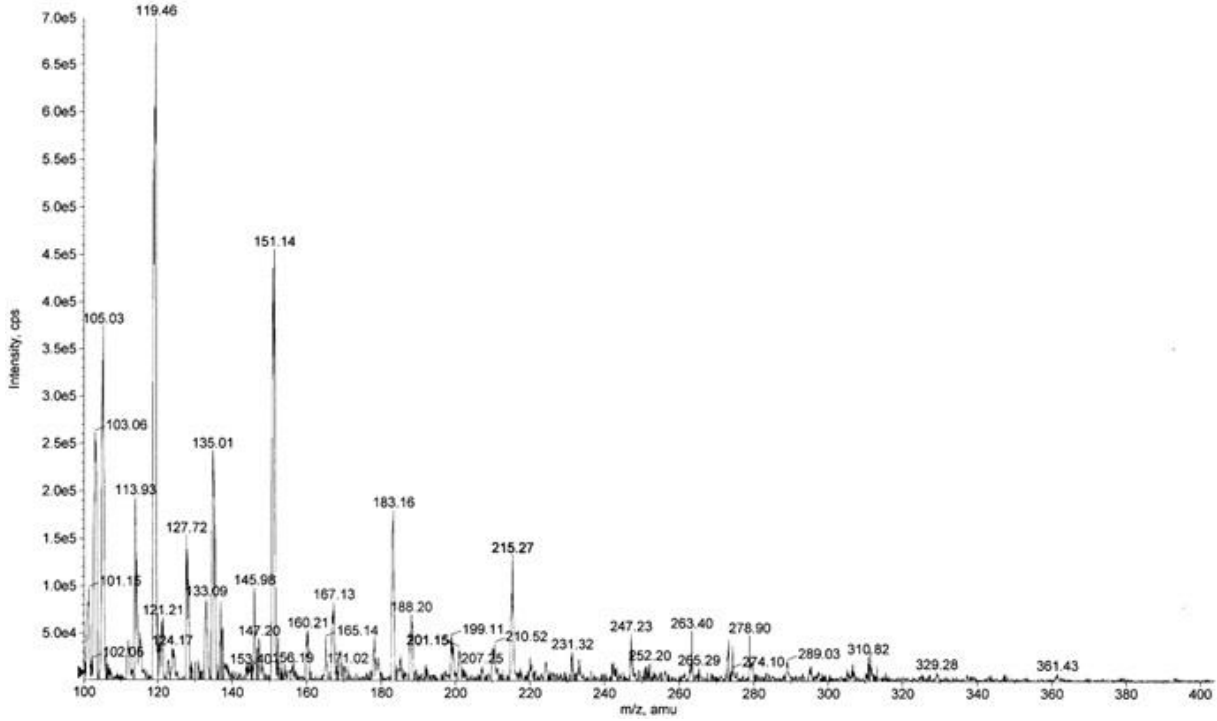
Appendix 40. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with H_2O_2 in acetic acid (+Q).



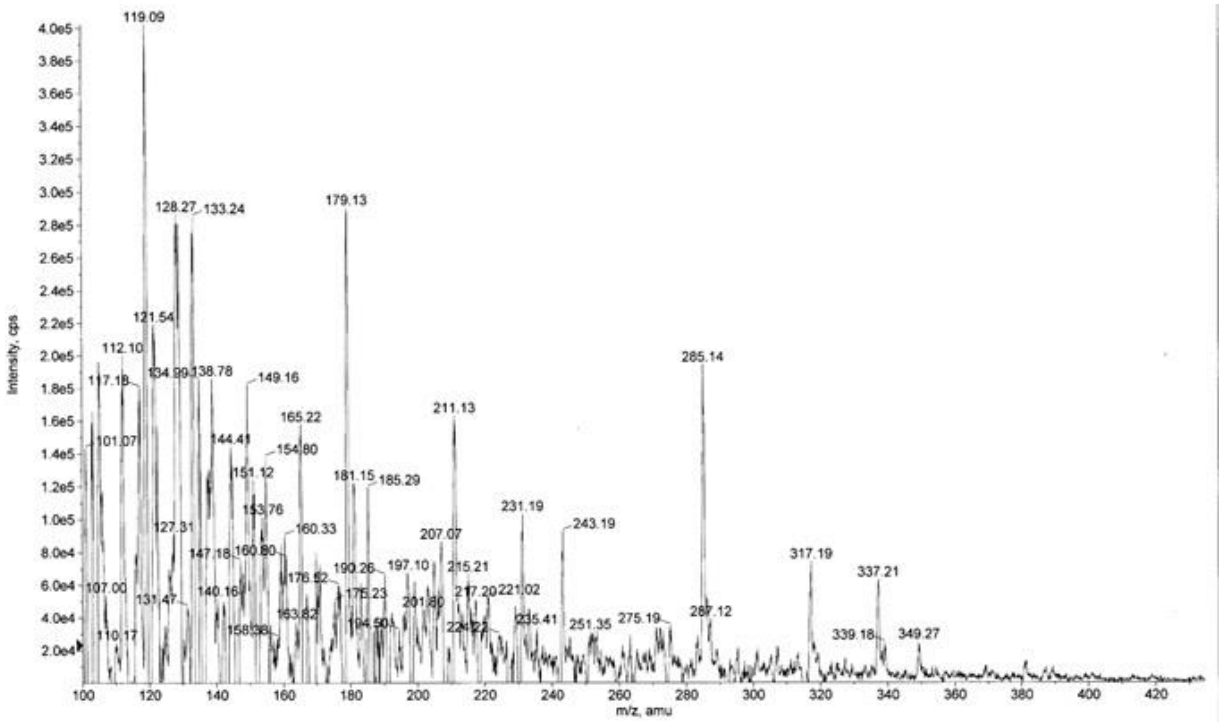
Appendix 41. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with H_2O_2 in acetic acid (-Q).



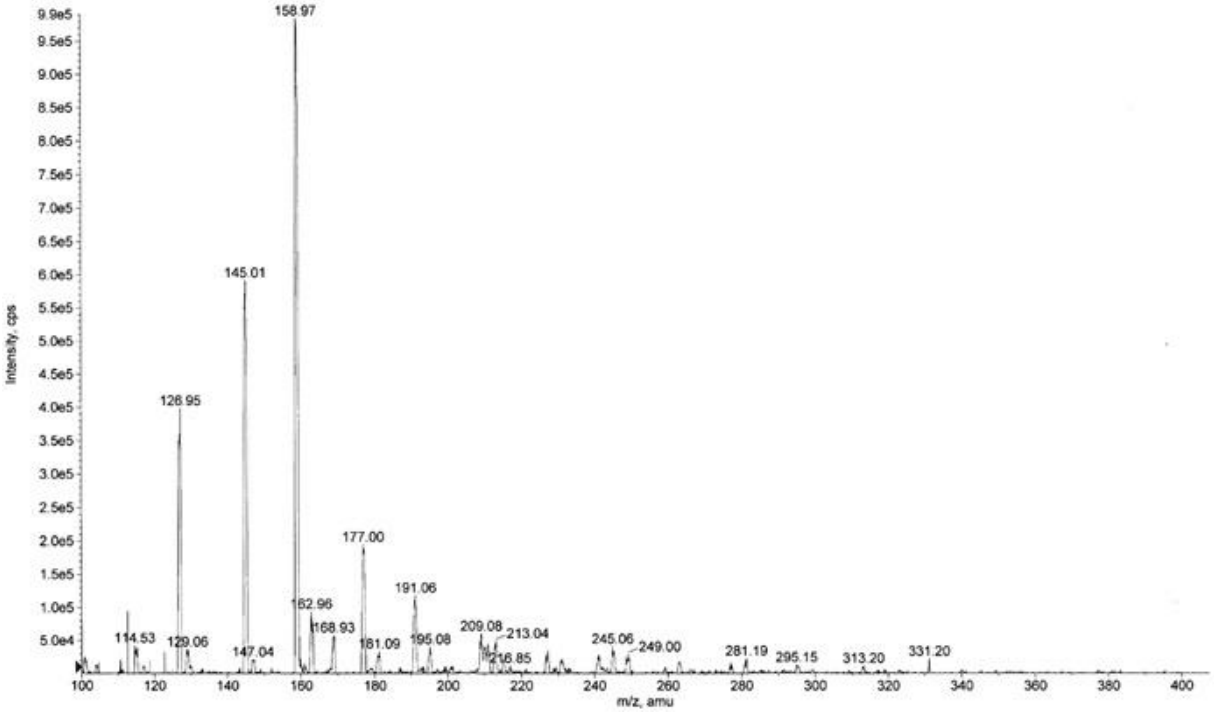
Appendix 42. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with cumol hydroxyl peroxide.



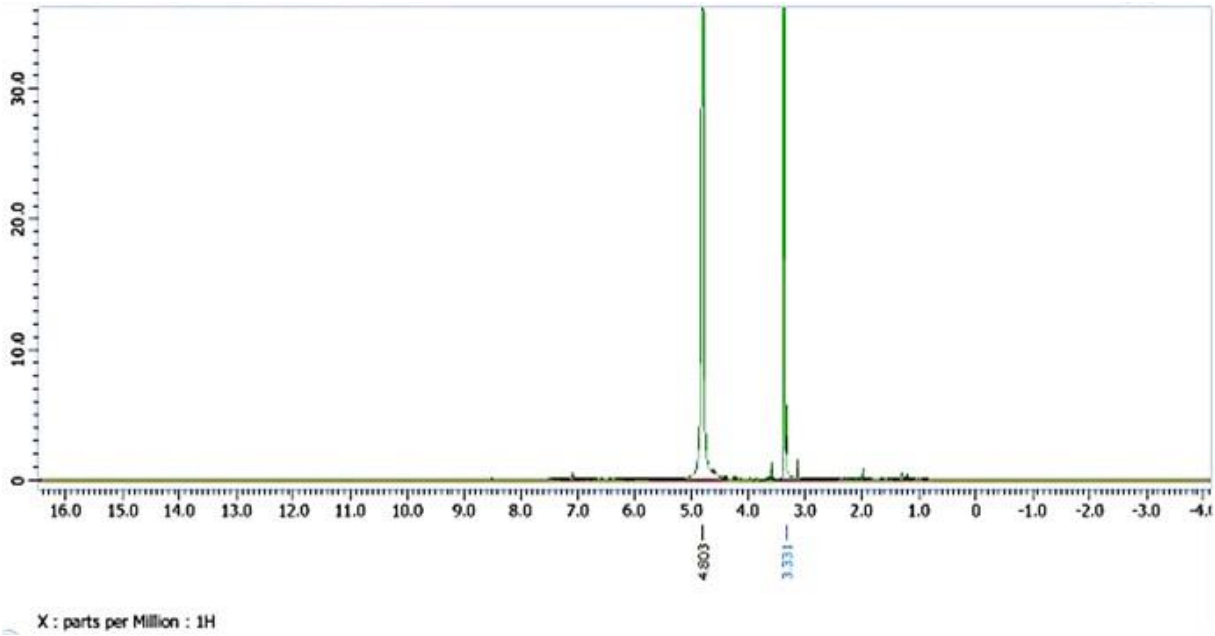
Appendix 43. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with MMPP



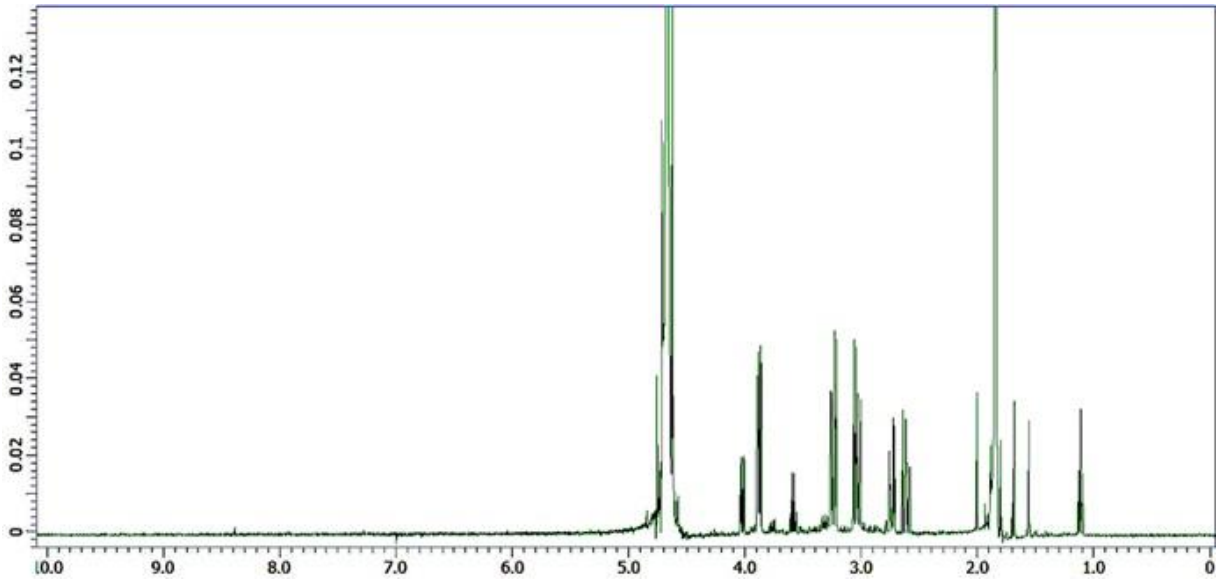
Appendix 44. ESI-MS analysis of oxidation of S-(N-methylpyrrol-2-yl)cysteine with TPAC.



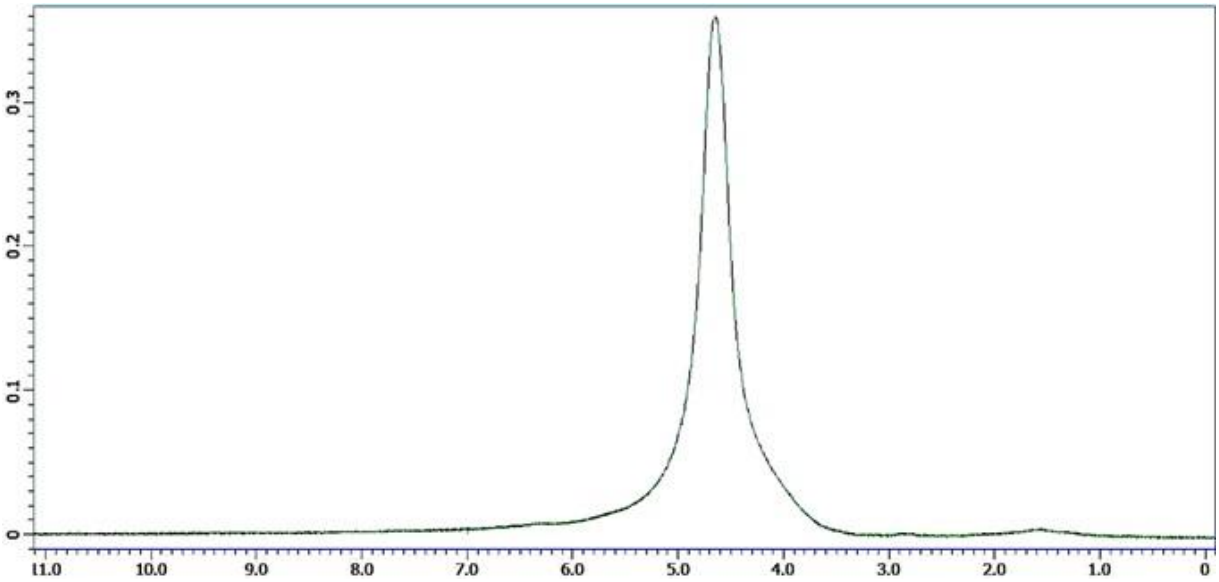
Appendix 45. ^{1}H NMR spectrum of S-(N-Bocpyrrol-2-yl)cysteine.



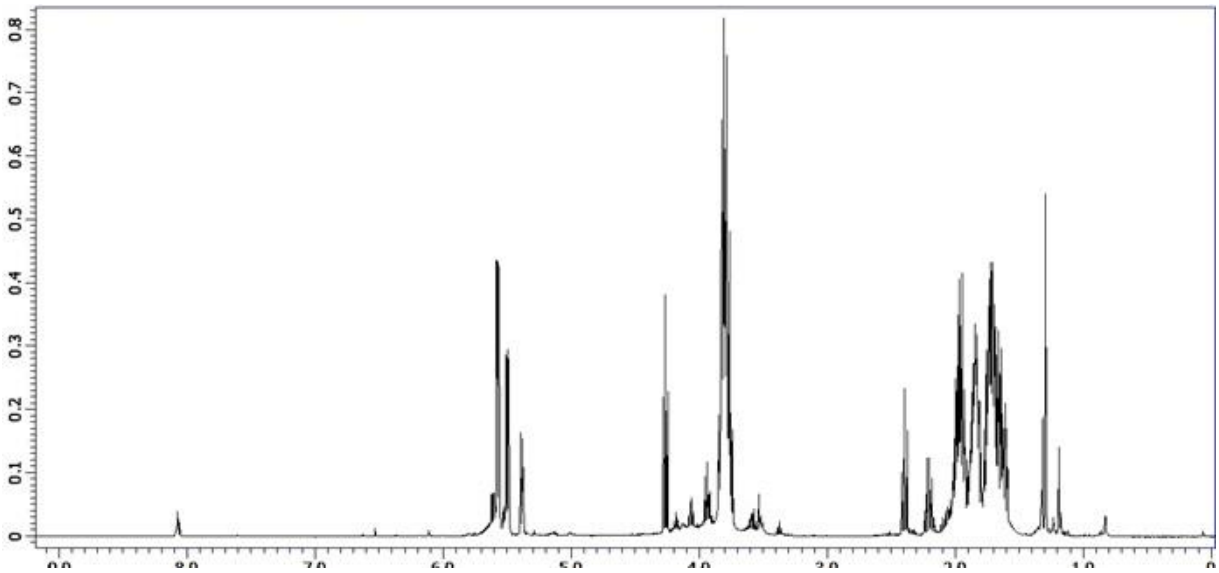
Appendix 46. ^{1}H-NMR analysis of S-(3-furanyl)cysteine.



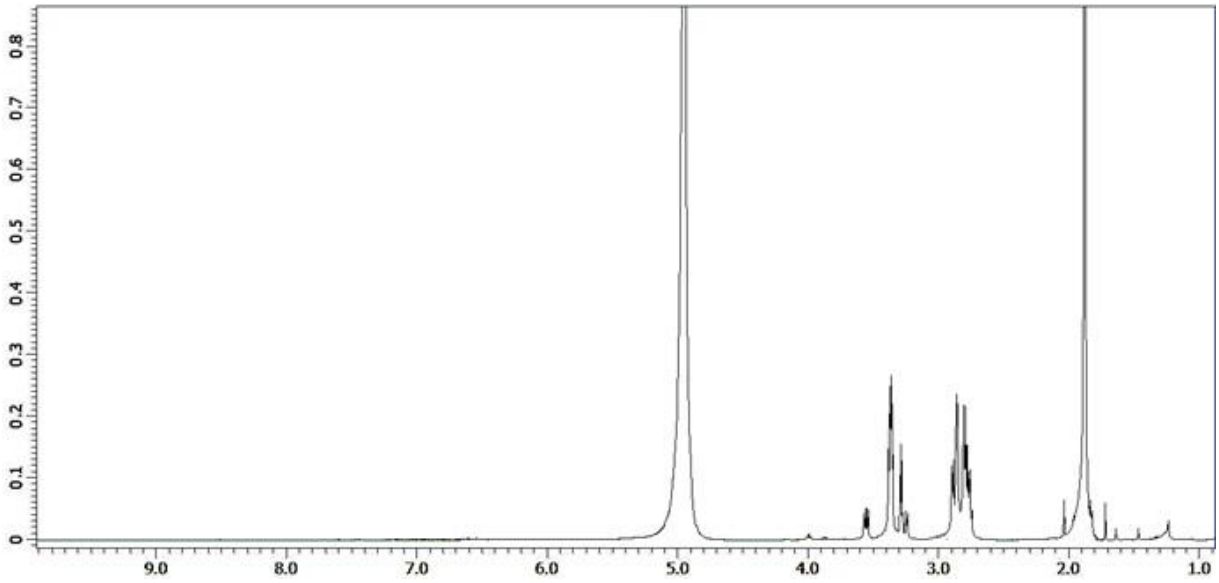
Appendix 47. ^{1}H-NMR spectrum of pyrrole-2-sulfonylchloride



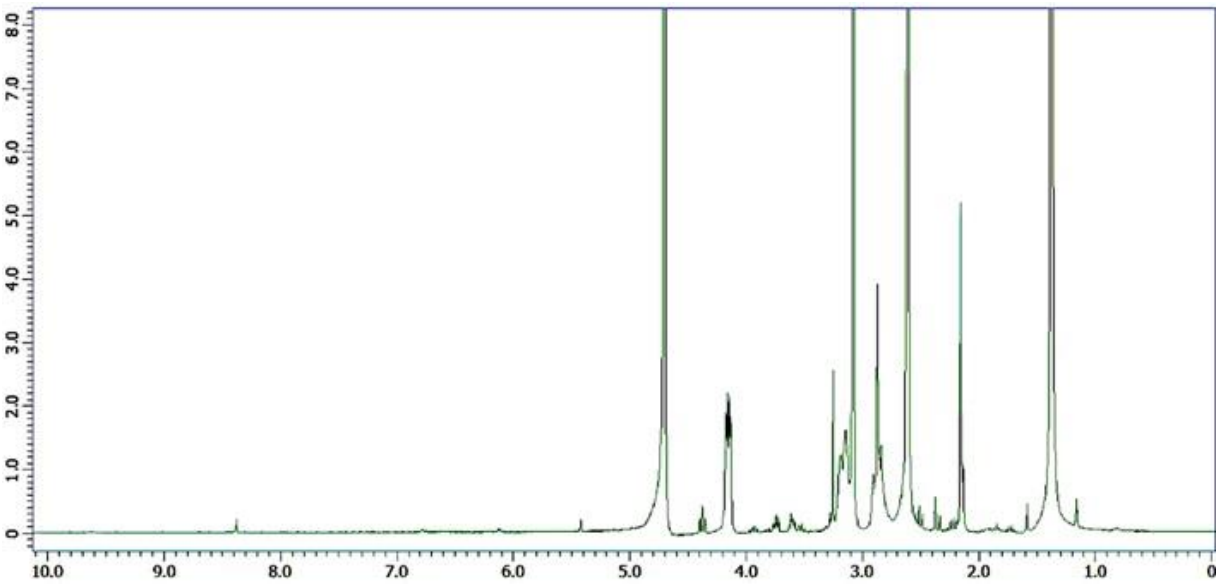
Appendix 48. ^{1}H-NMR spectrum of furan-3-sulfonyl chloride.



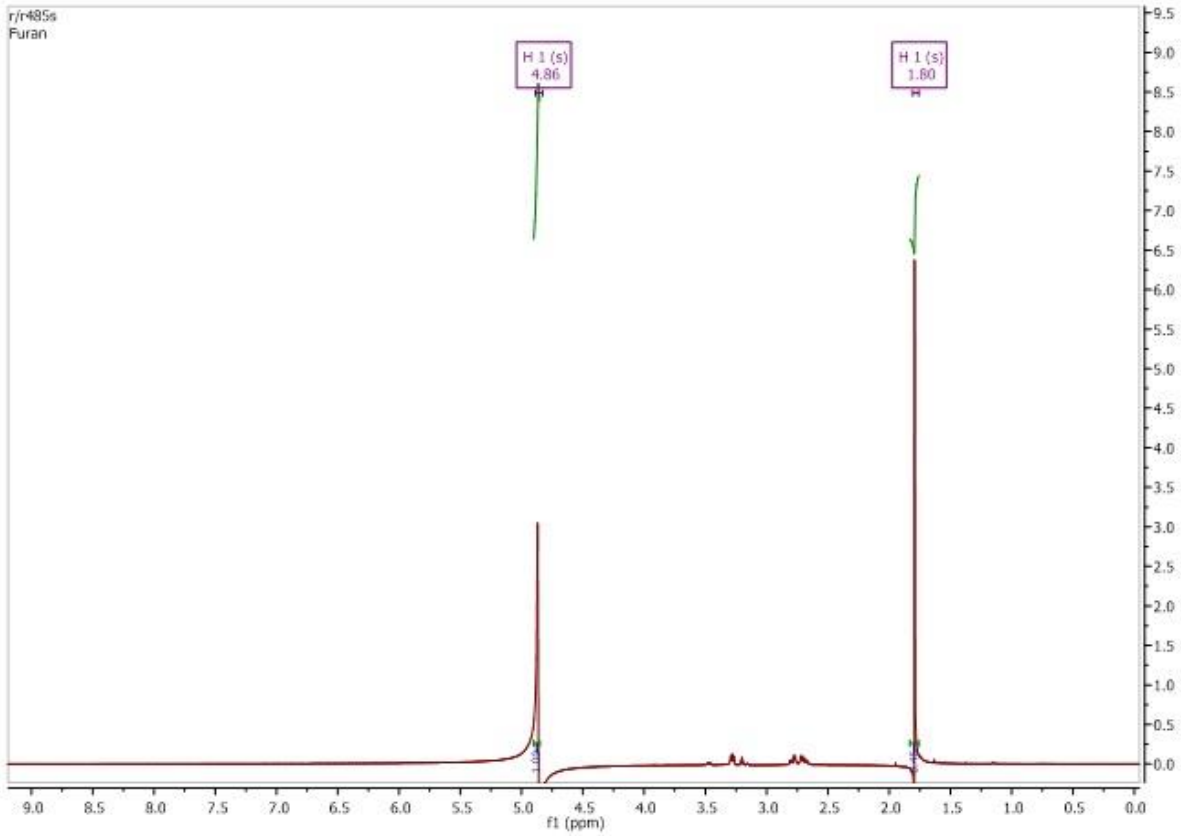
Appendix 49. ^{1}H-NMR spectrum of S-(2-furanyl)cysteine.



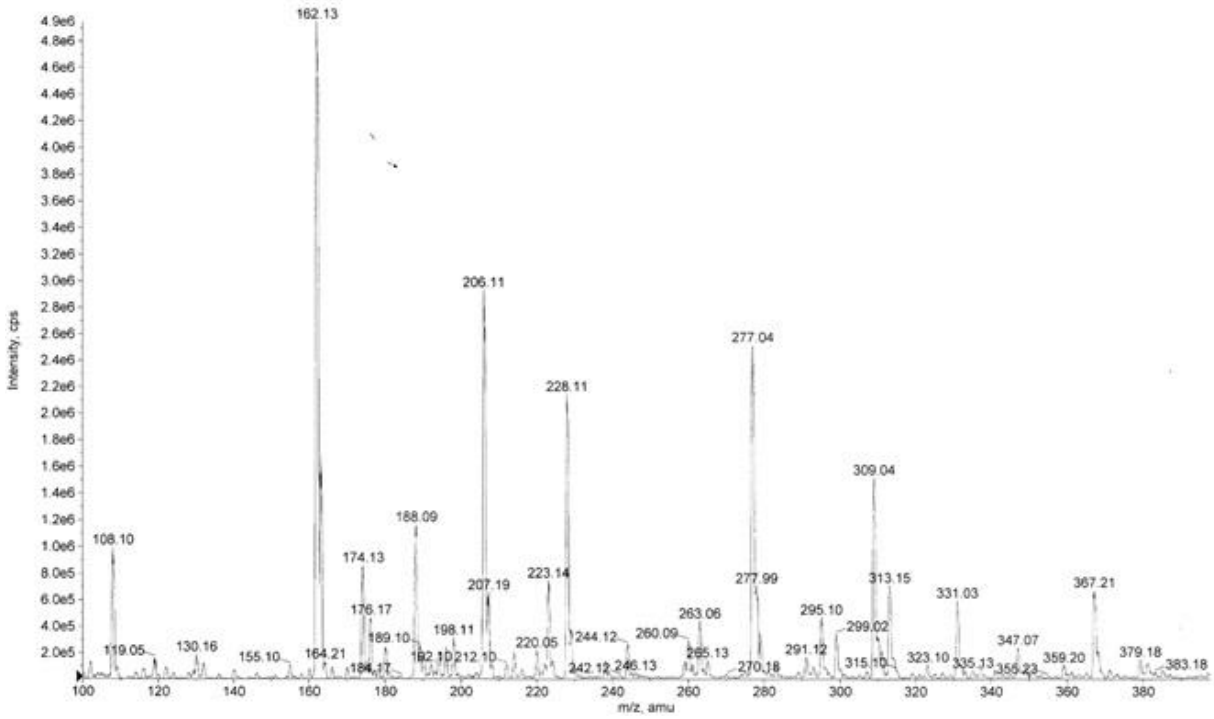
Appendix 50. ^{1}H NMR spectrum of S-(3-thienyl)cysteine.



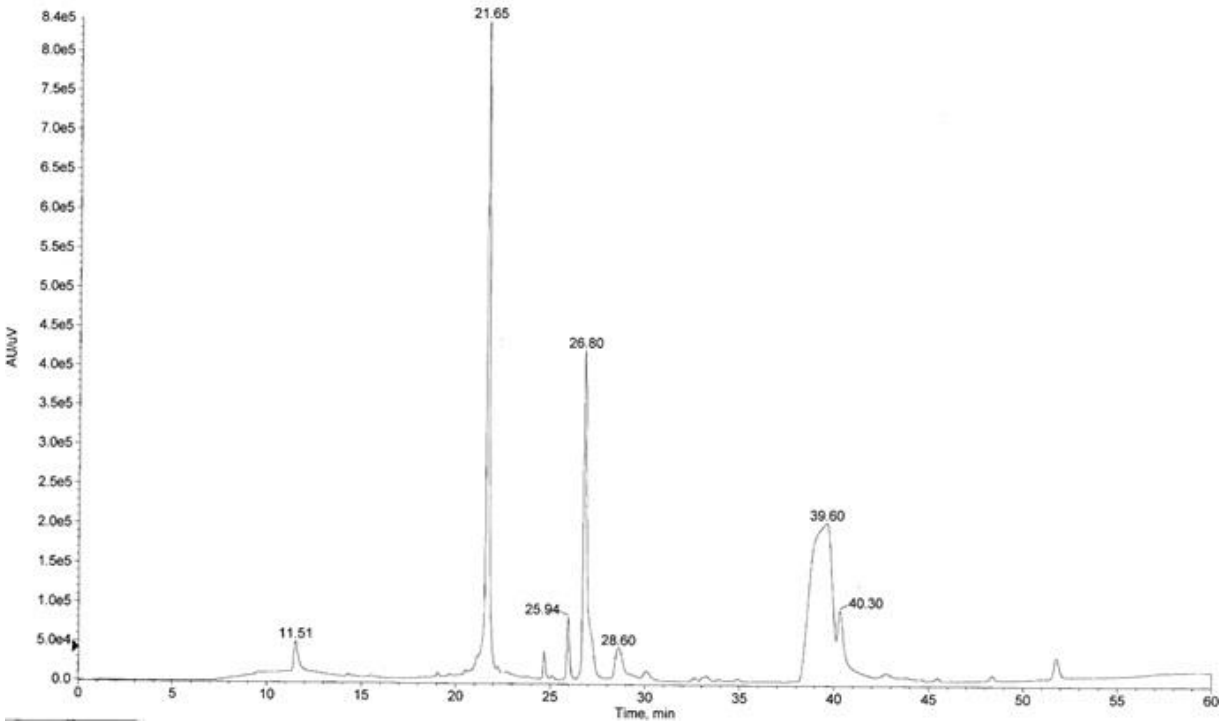
Appendix 51. ^{1}H-NMR spectrum of S-(3-furanyl)cysteine



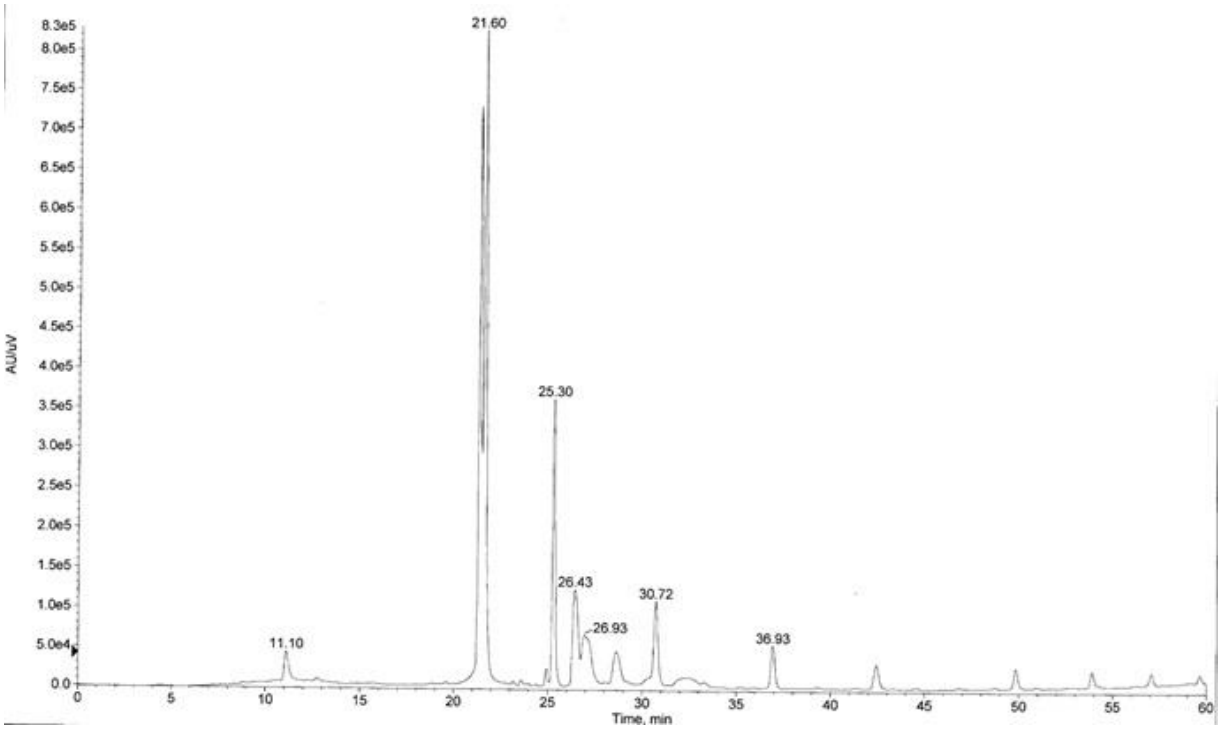
Appendix 52. ESI-MS analysis of catalytic transfer hydrogenation of S-(N-benzylpyrrol-2-yl)cysteine-S-oxide.



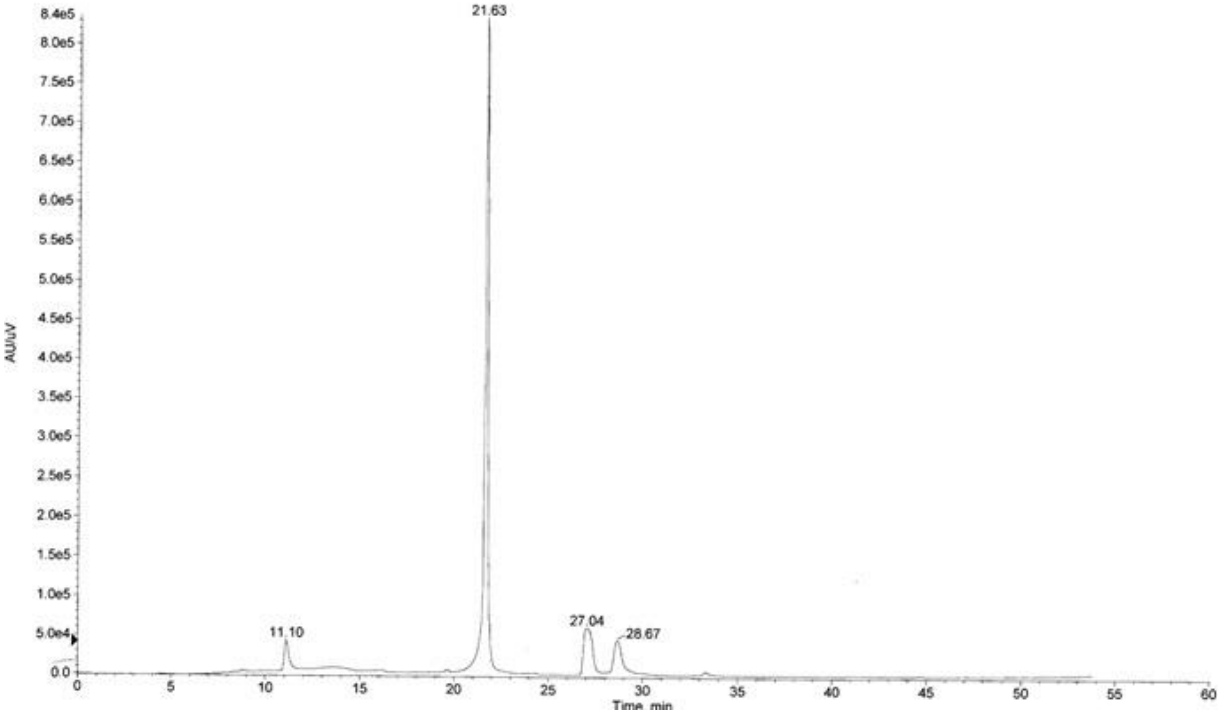
Appendix 53. HPLC chromatogram of the allinase reaction on S-(2-thienyl)cysteine-S-oxide after 2 h.



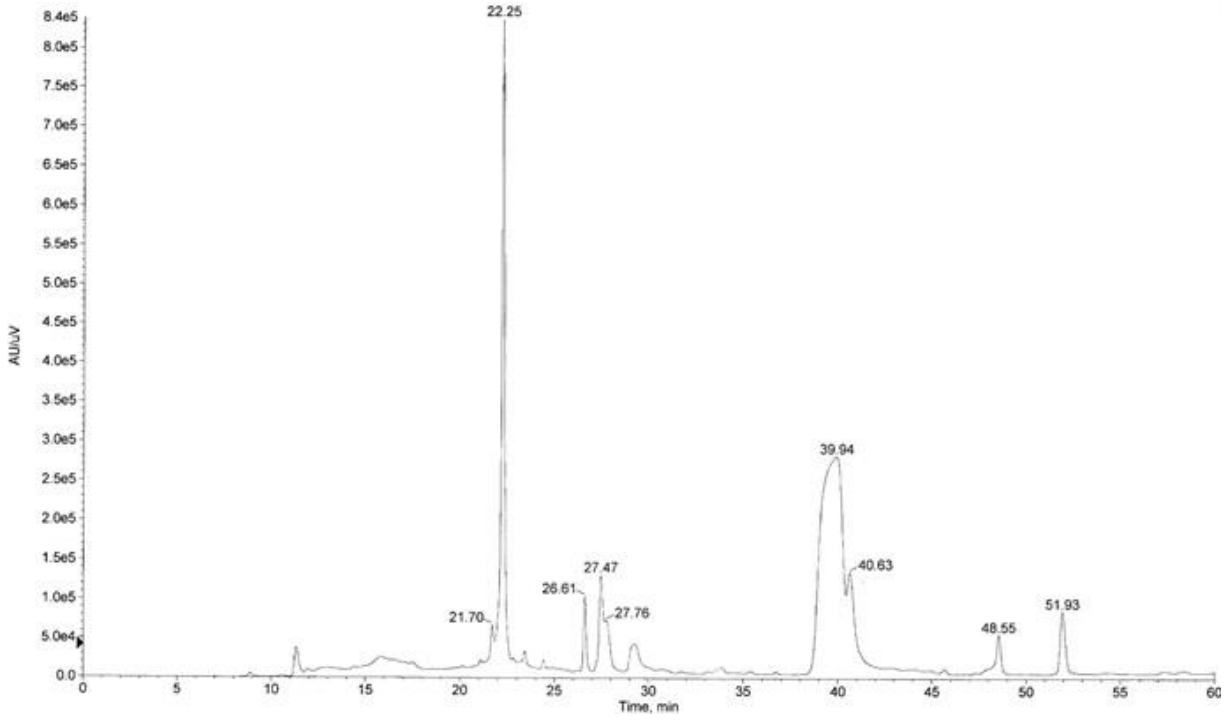
Appendix 54. HPLC chromatogram of the allinase reaction on alliin as positive control after 2h.



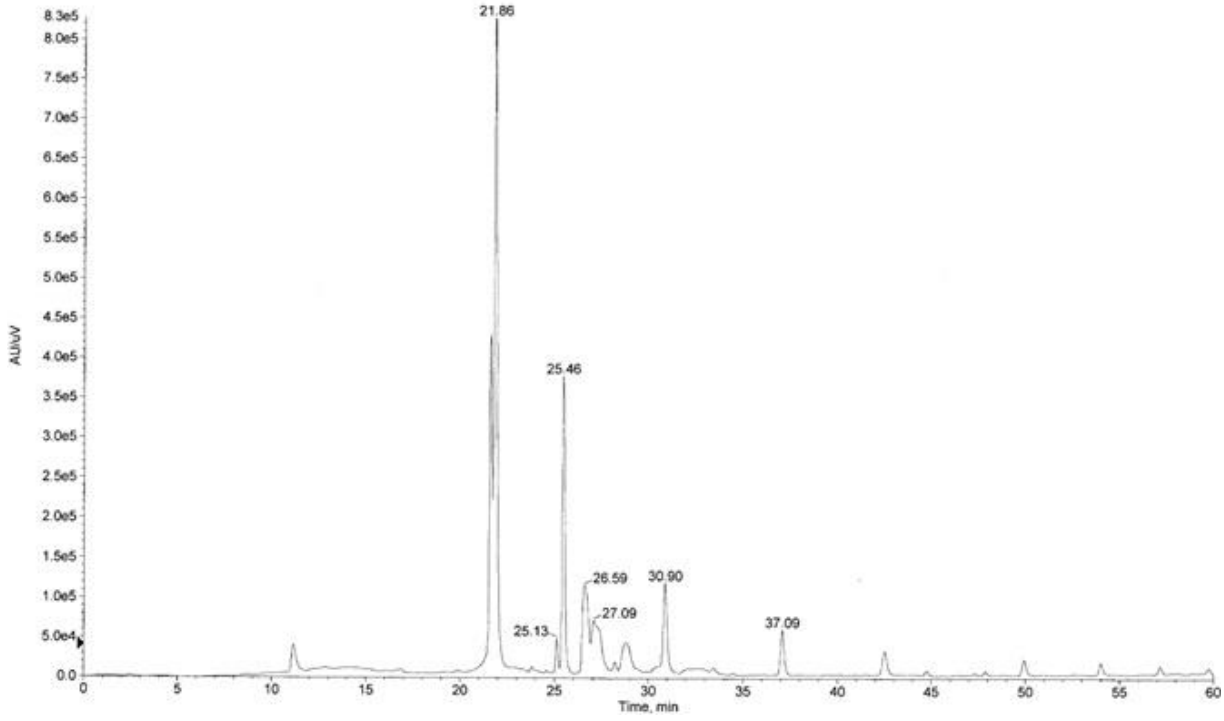
Appendix 55. HPLC chromatogram of the allinase as negative control after 2h.



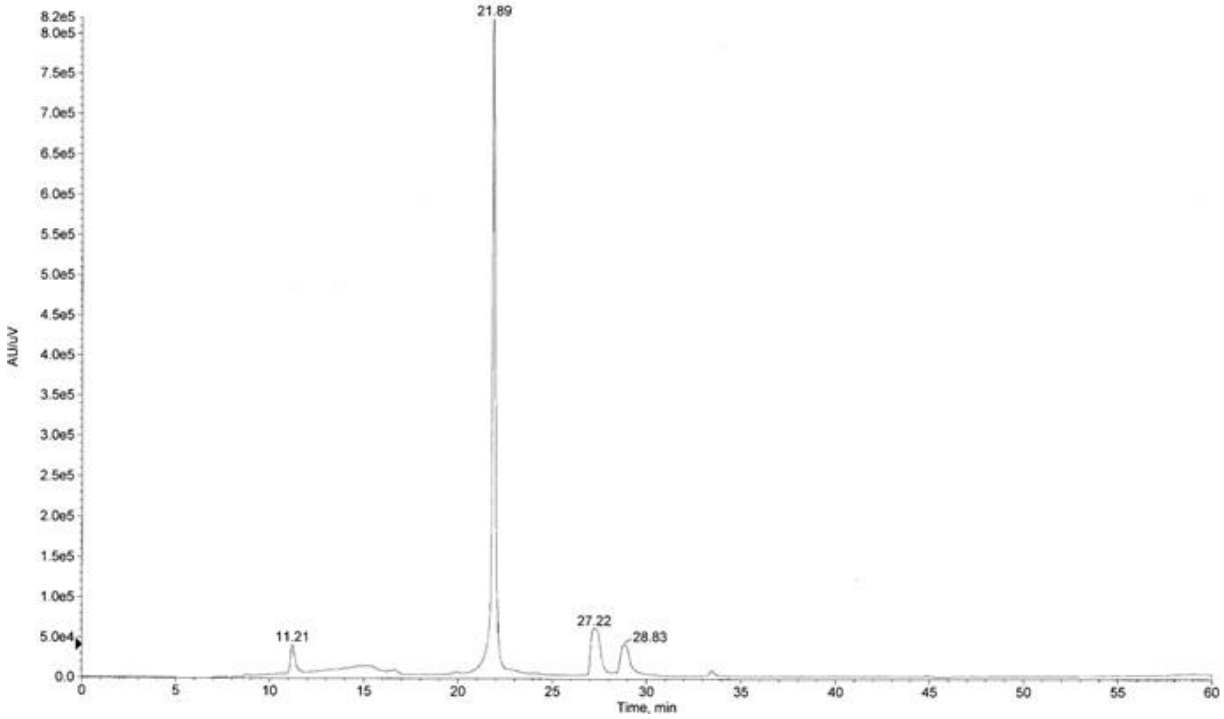
Appendix 56. HPLC chromatogram of the alliinase reaction on S-(2-thienyl)cysteine-S-oxide after 4h.



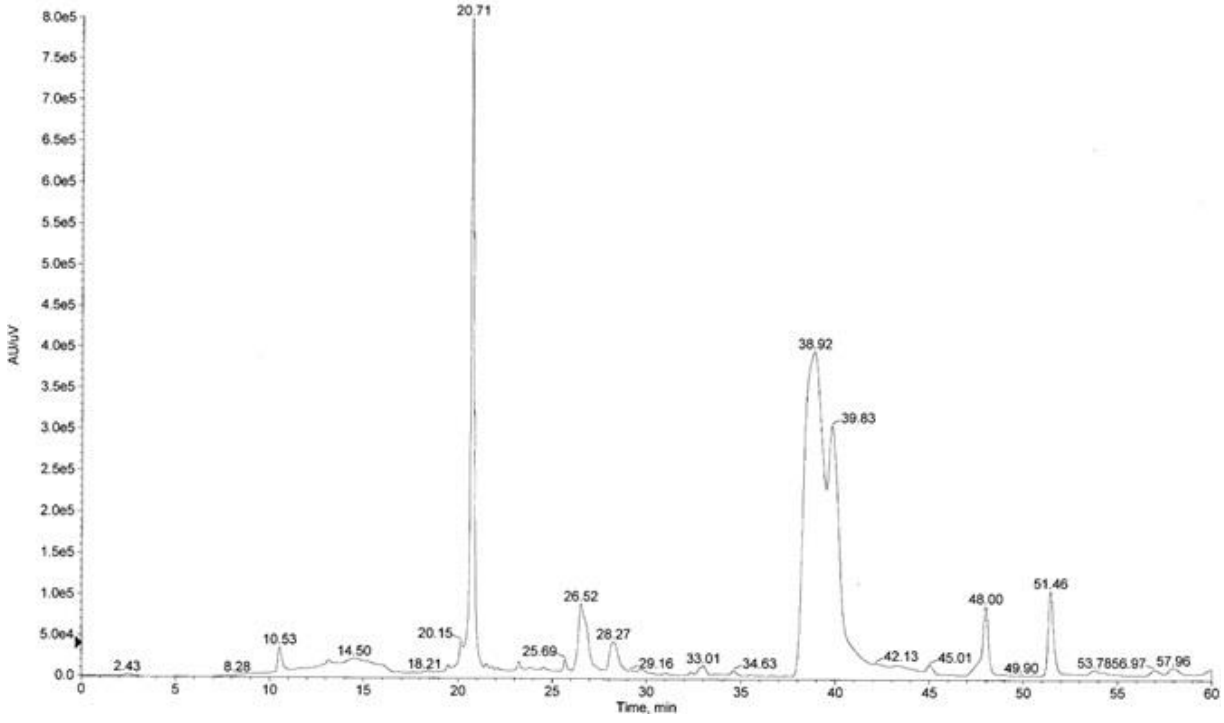
Appendix 57. HPLC chromatogram of the allinase reaction on alliin as positive control after 4h.



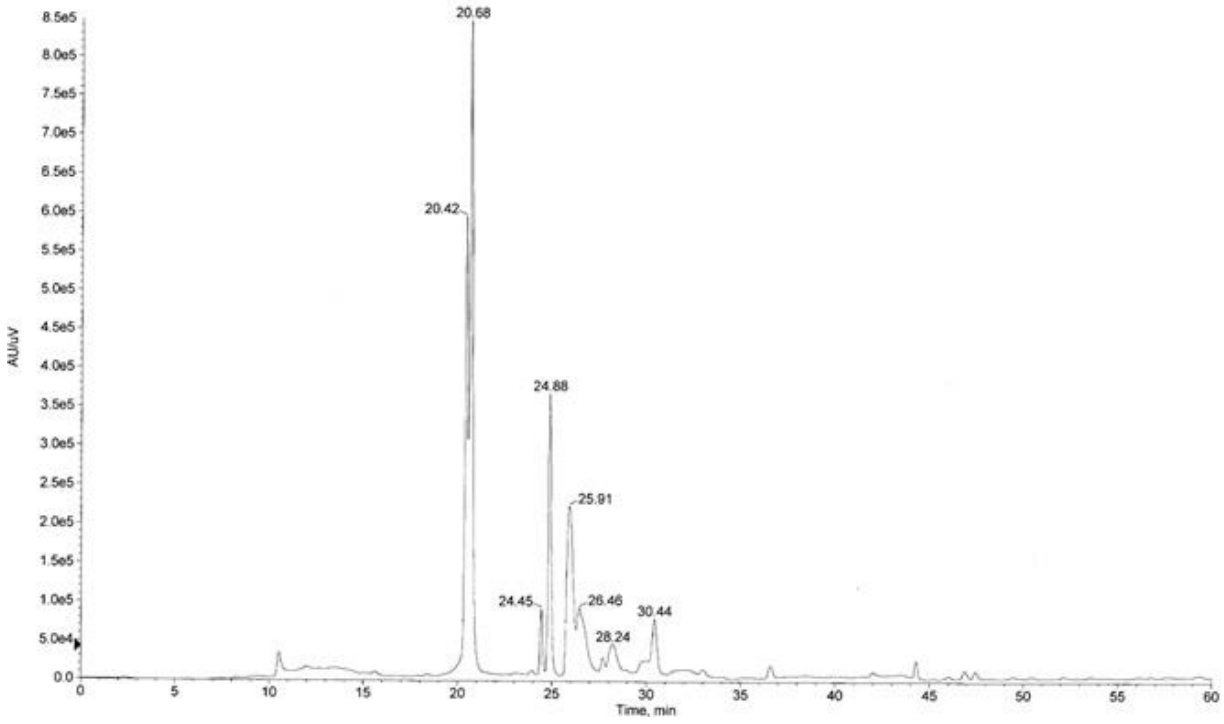
Appendix 58. HPLC chromatogram of alliinase as negative control after 4h



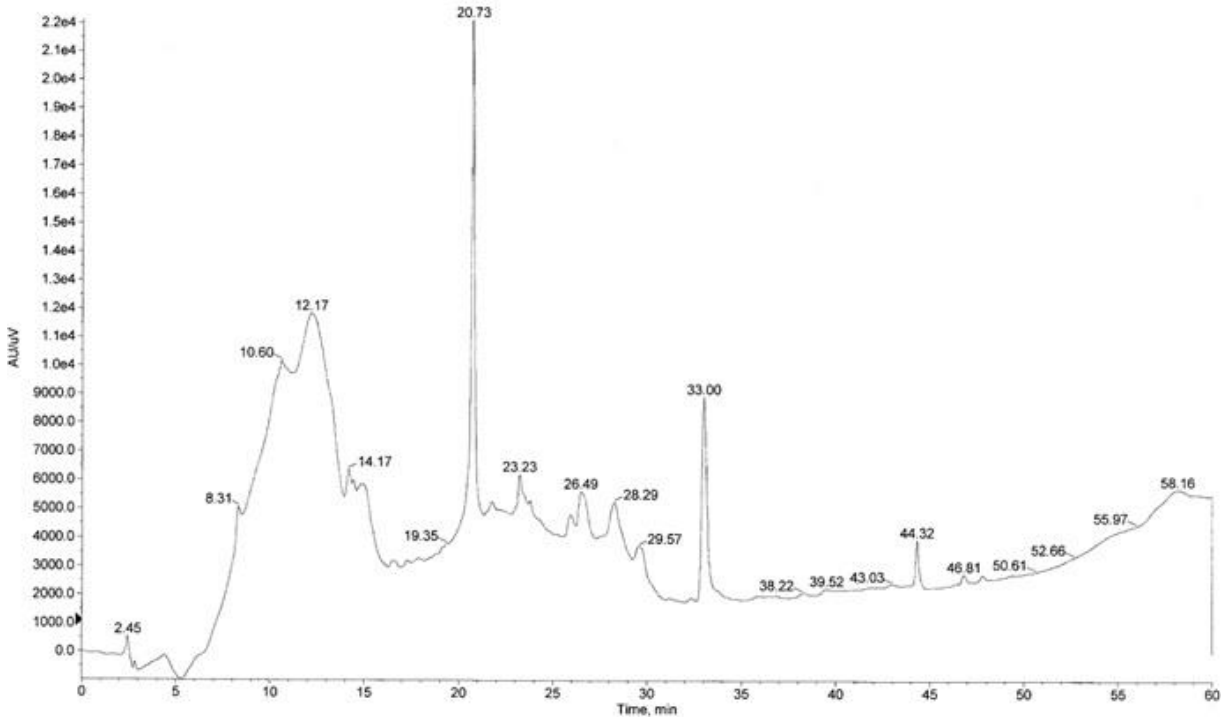
Appendix 59. HPLC chromatogram of the alliinase reaction on S-(2-thienyl)cysteine-S-oxide after 6h



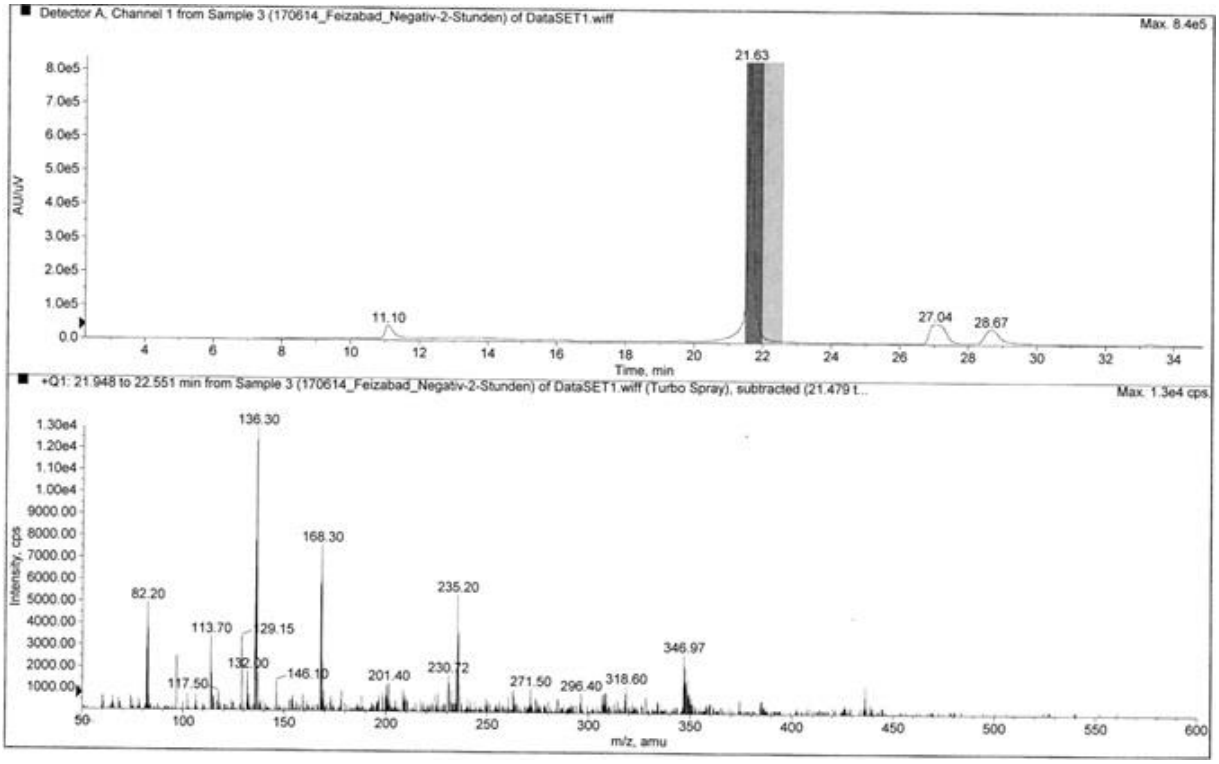
Appendix 60. HPLC chromatogram of the allinase reaction on alliin as positive control after 6h



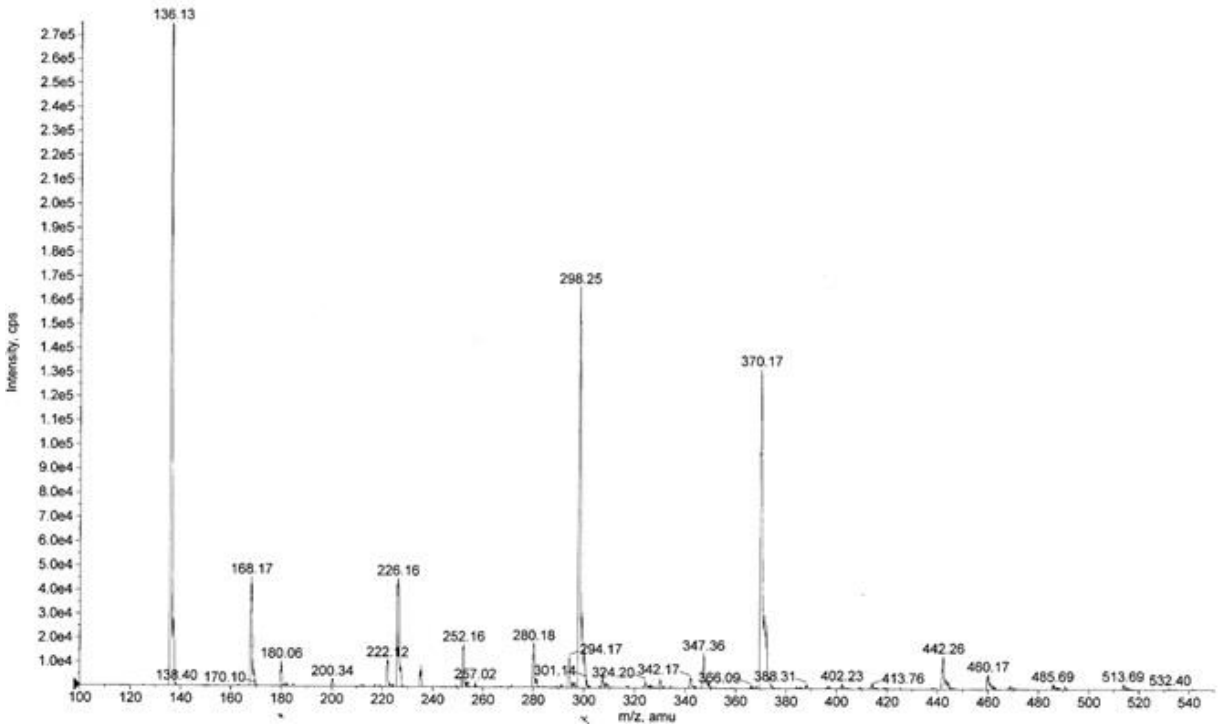
Appendix 61. HPLC chromatogram of alliinase as negative control after 6h.



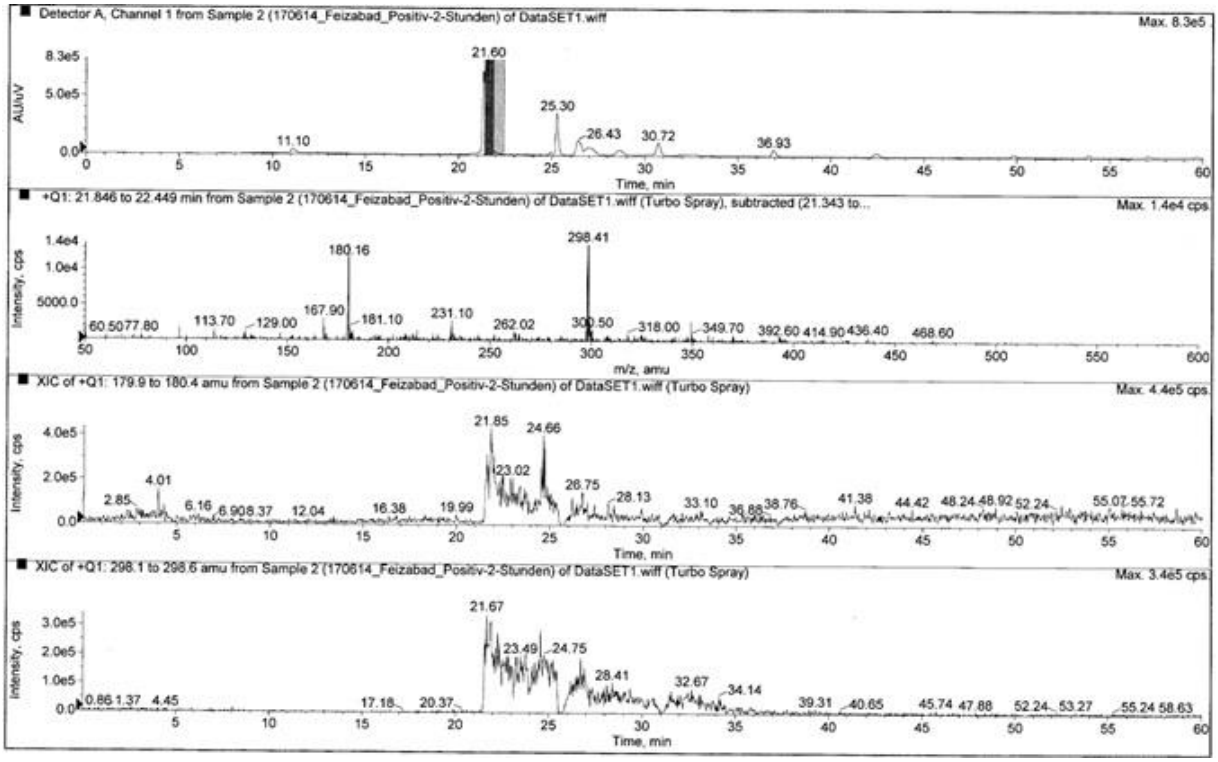
Appendix 62. HPLC-MS chromatogram of negative control after 2h for the peak with molecular weight 135 g/mol.



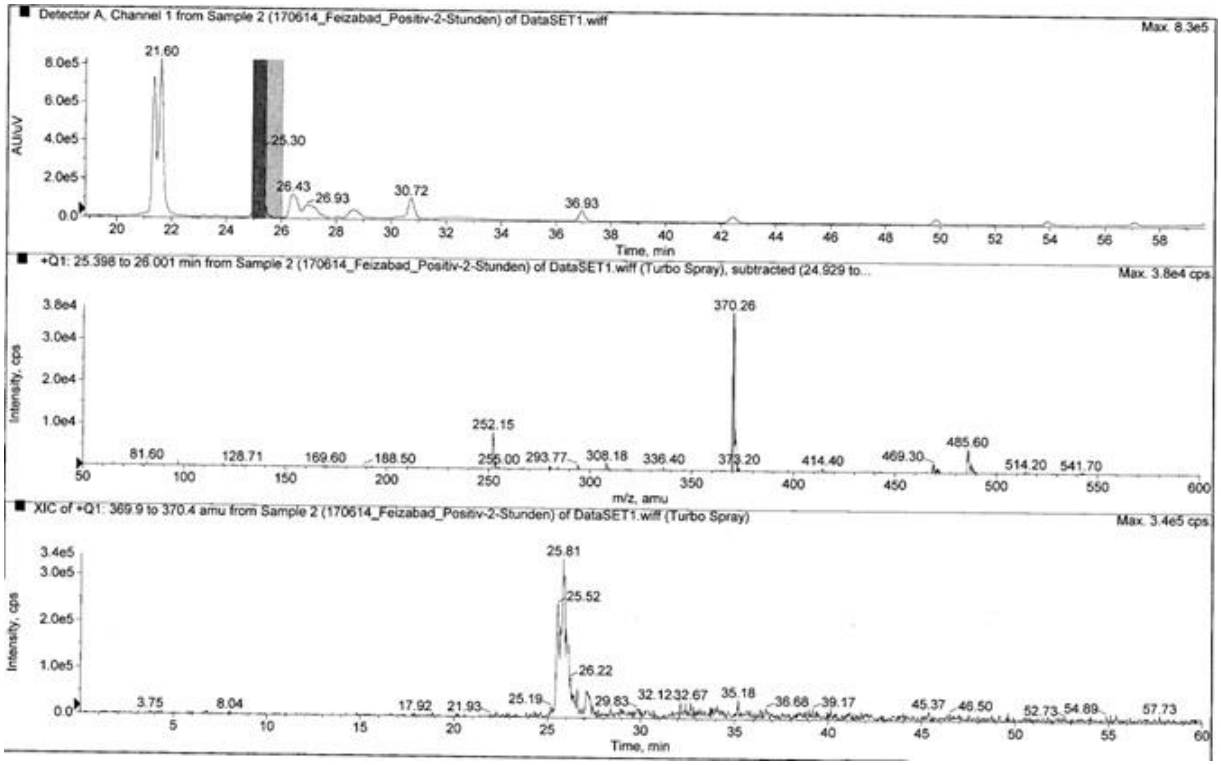
Appendix 63. +Q-MS of the allinase reaction on alliin as positive control positive control after 2h.



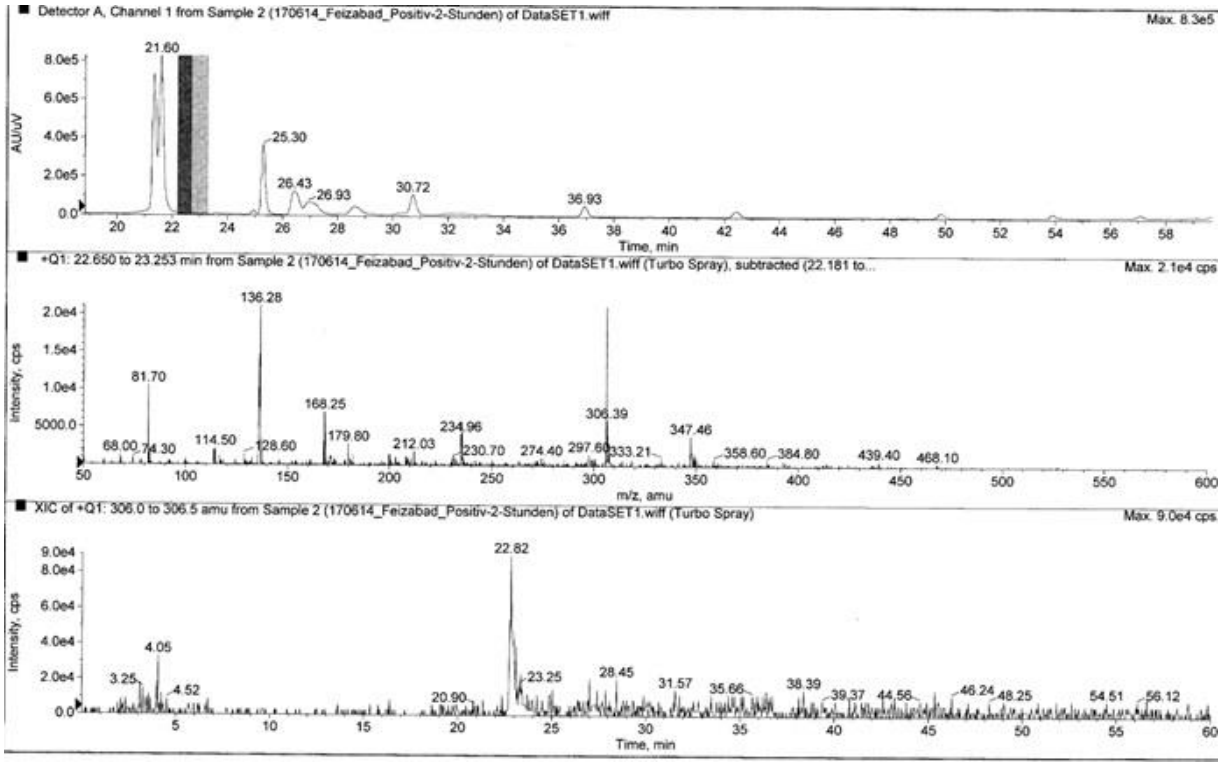
Appendix 64. HPLC chromatogram of the allinase reaction on alliin as positive control after 2h for allicin.



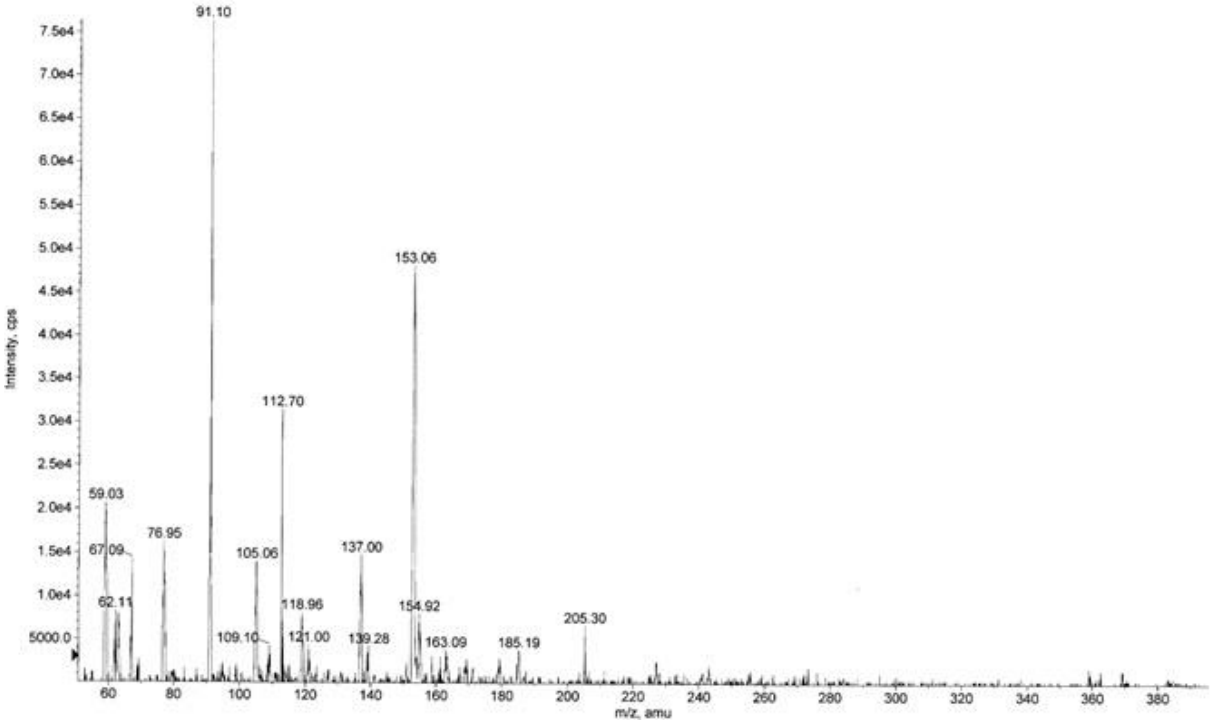
Appendix 65. HPLC chromatogram of the allinase reaction on alliin as positive control after 2h for ajoene.



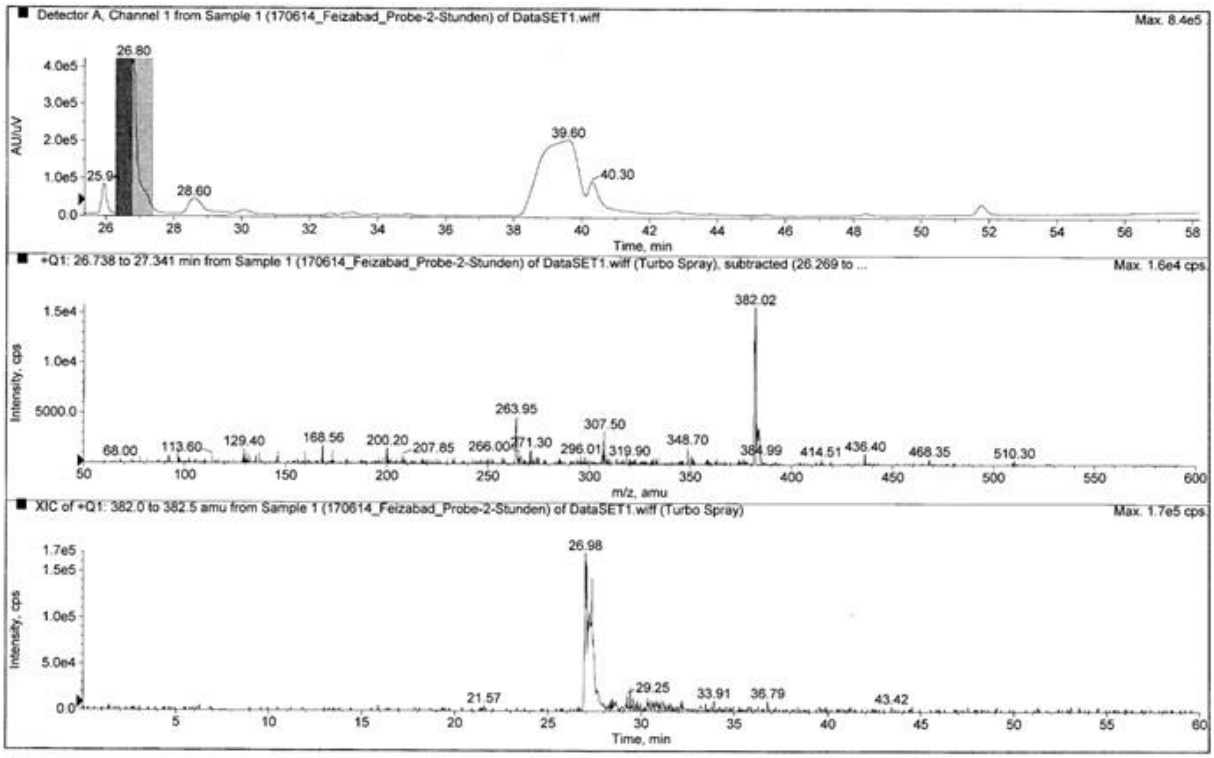
Appendix 66. HPLC chromatogram of the allinase reaction on alliin as positive control after 2h for the peak with molecular weight 135 g/mol



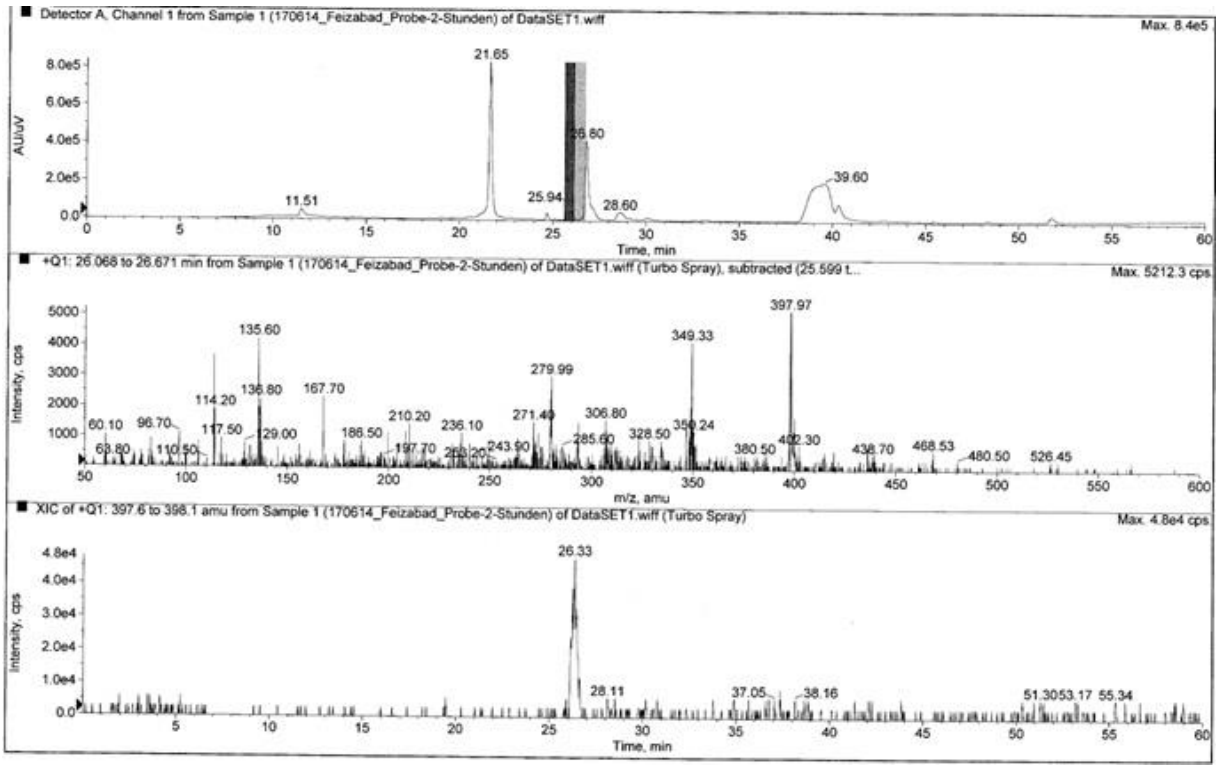
Appendix 67. -Q-MS of HPLC chromatogram of the allinase reaction on alliin as positive control after 2h



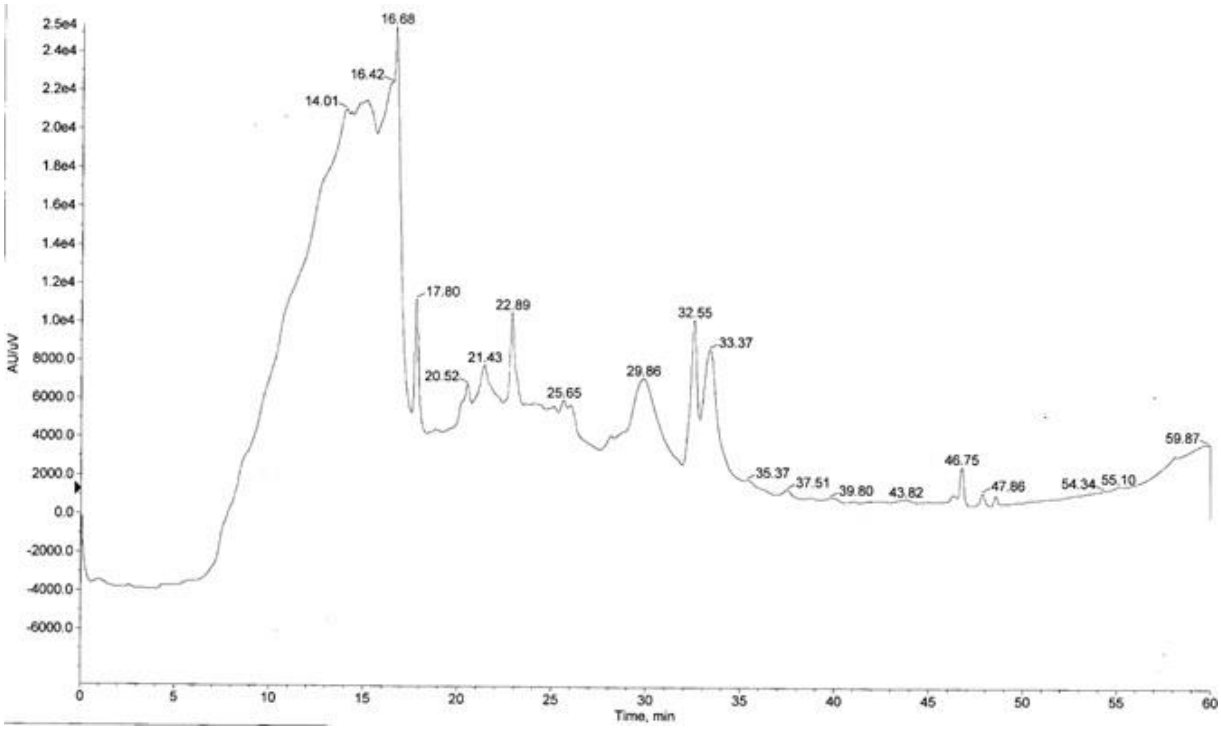
Appendix 68. HPLC-MS chromatogram of disulfide-S-oxide at 26.98 min after 2h.



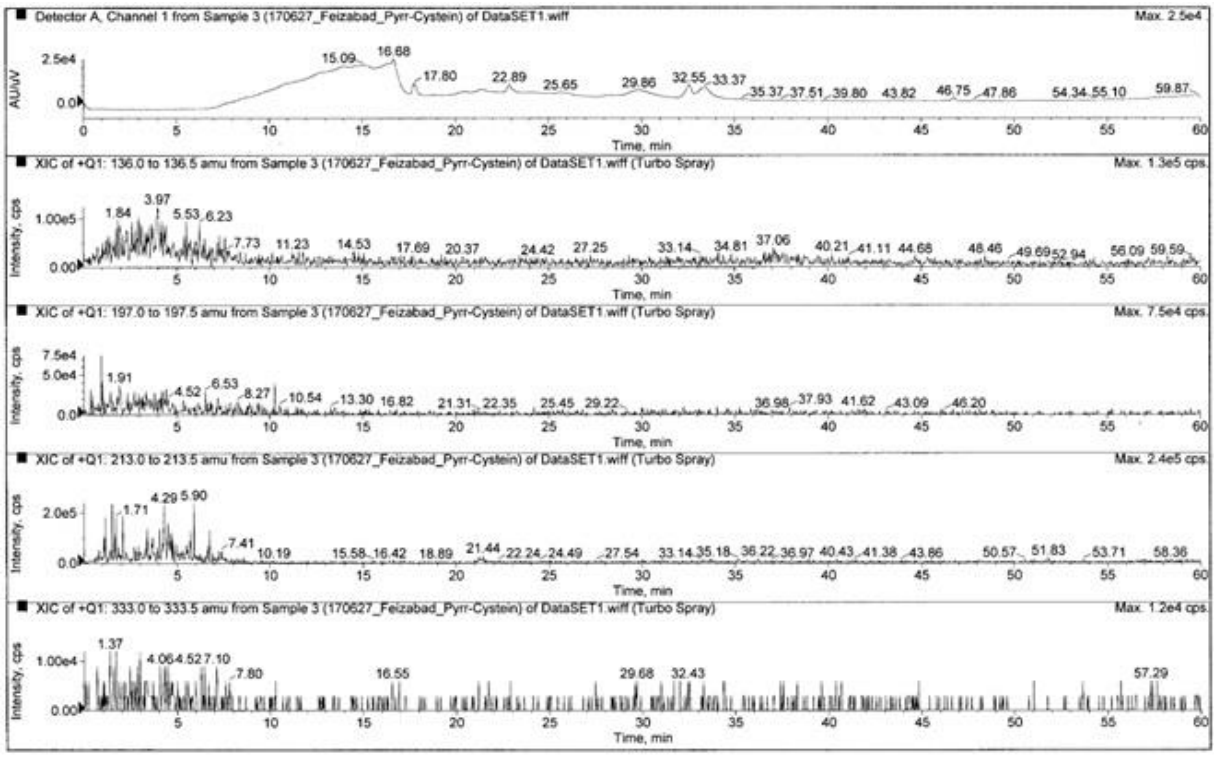
Appendix 69. HPLC-MS chromatogram of disulfide-S-S´-dioxide at 26.33 min after 2h.



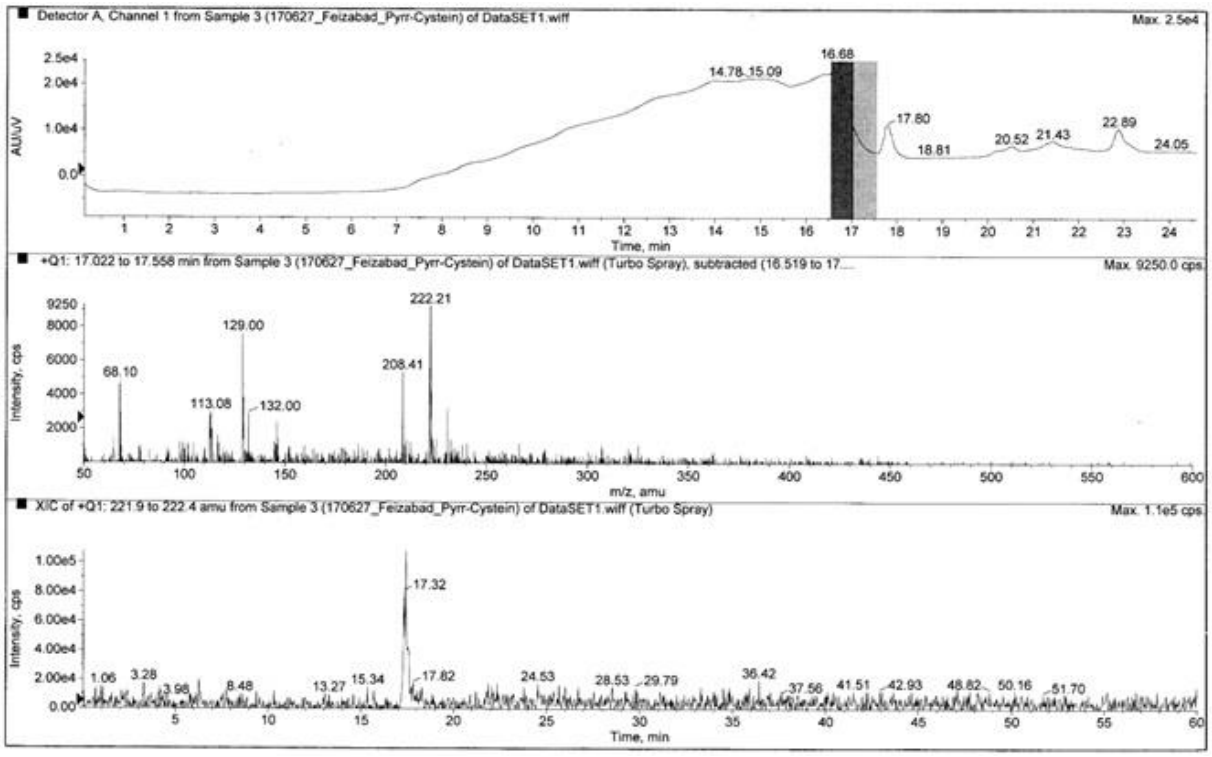
Appendix 70. HPLC chromatogram of alliinase reaction on S-(2-pyrrolyl)cysteine after 2h.



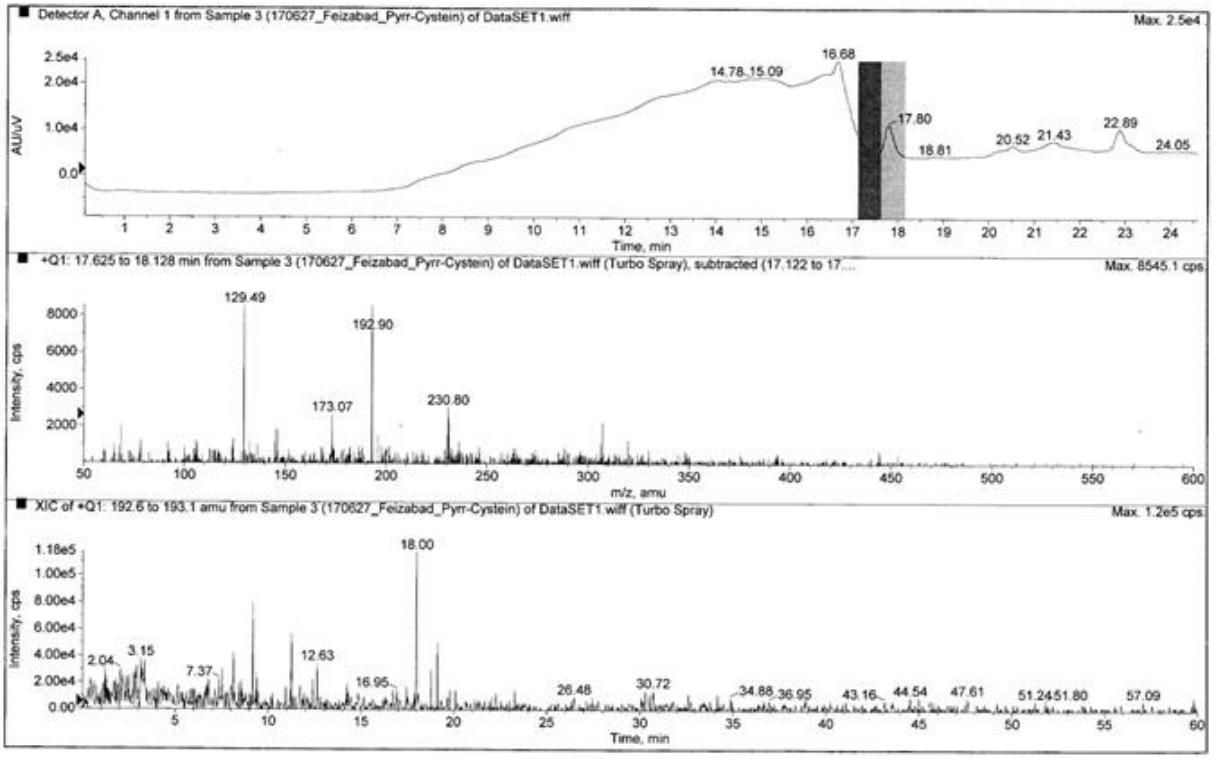
Appendix 71. HPLC-MS chromatogram of alliinase reaction on S-(2-pyrrolyl)cysteine after 2h



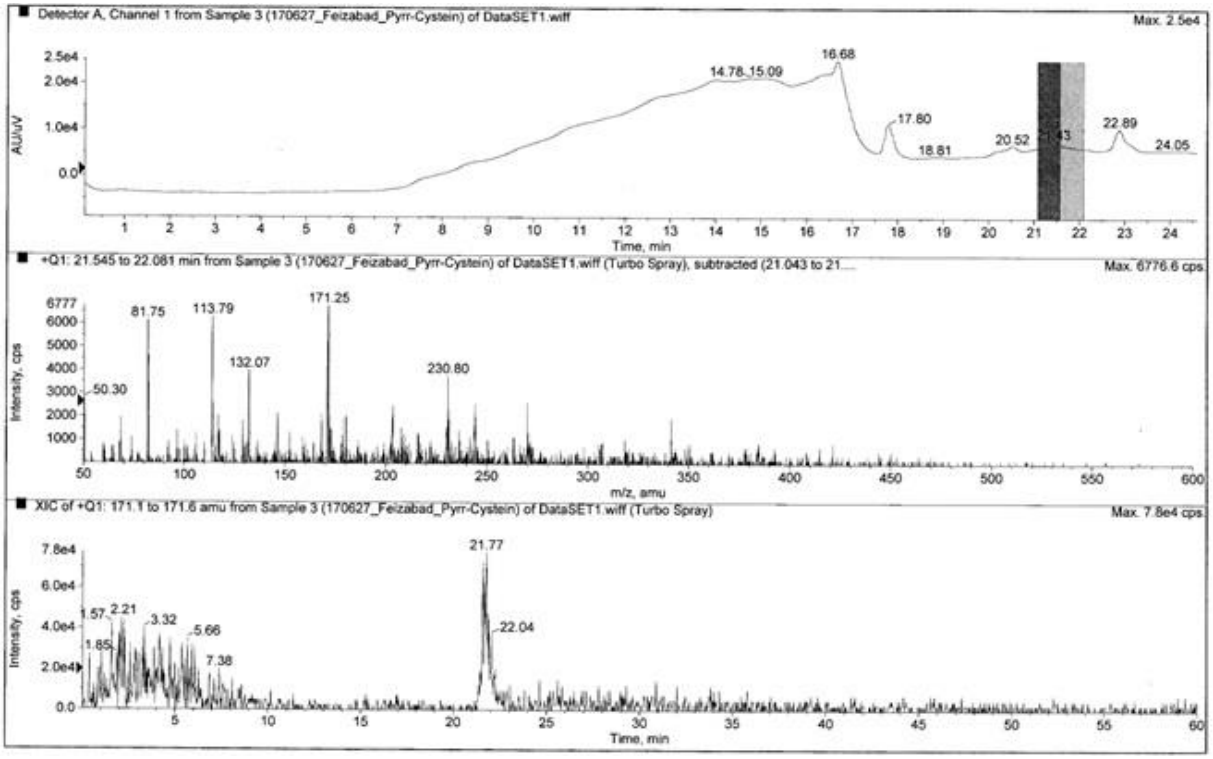
Appendix 72. HPLC-MS chromatogram of allinase reaction on the S-(2-pyrrolyl)cysteine at 17.32 min after 2h.



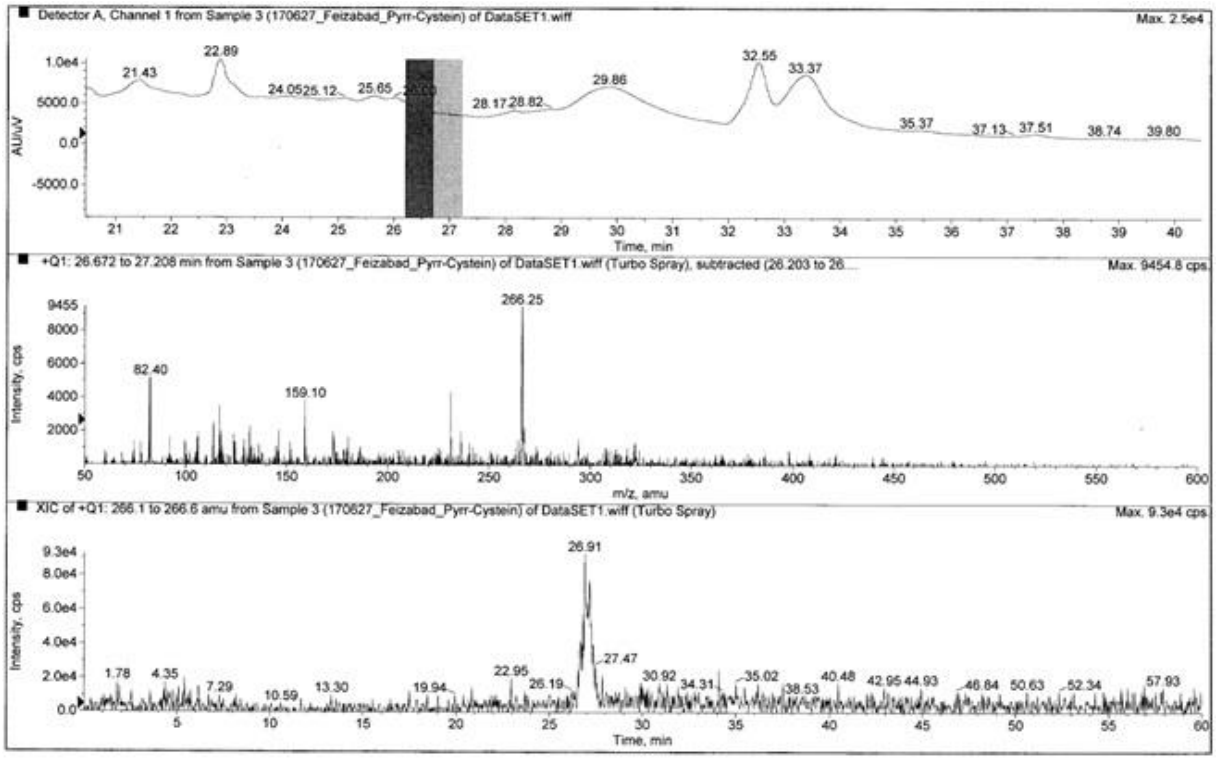
Appendix 73. HPLC-MS chromatogram of allinase reaction on S-(2-pyrrolyl)cysteine at 18 min after 2h.



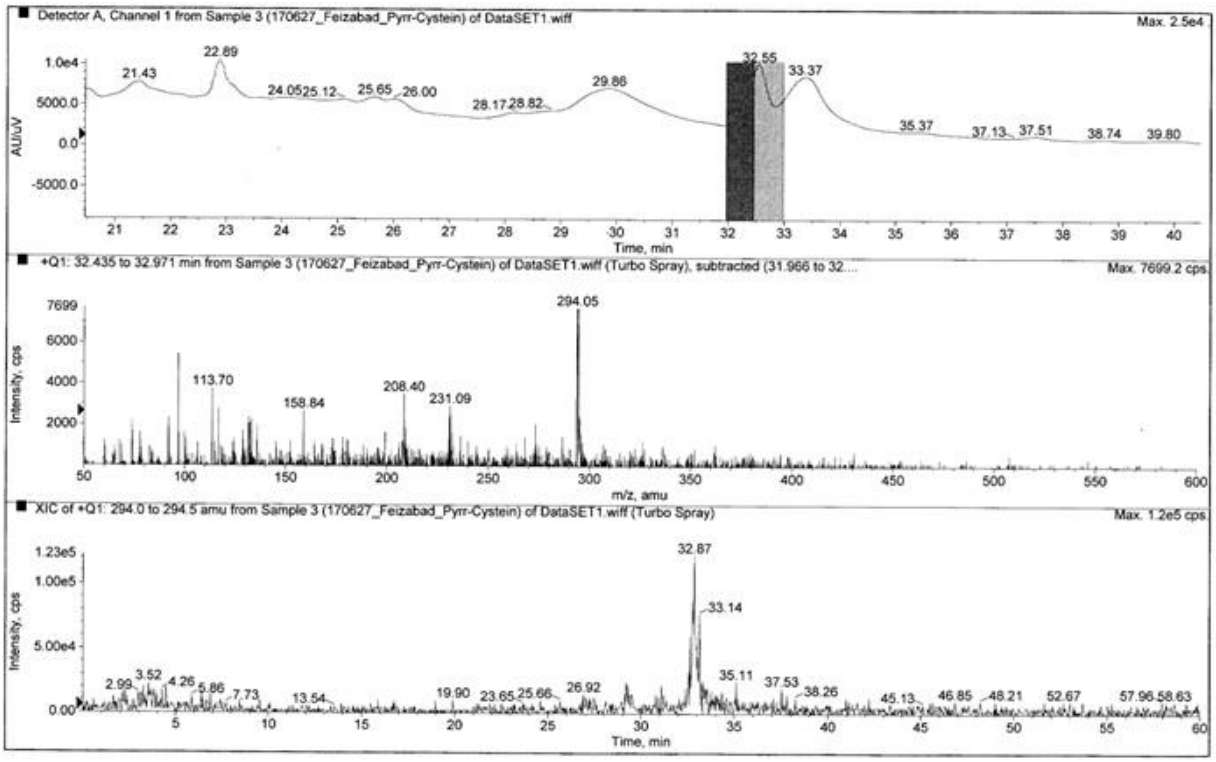
Appendix 74. HPLC-MS chromatogram of allinase reaction on S-(2-pyrrolyl)cysteine at 21.77 min after 2h.



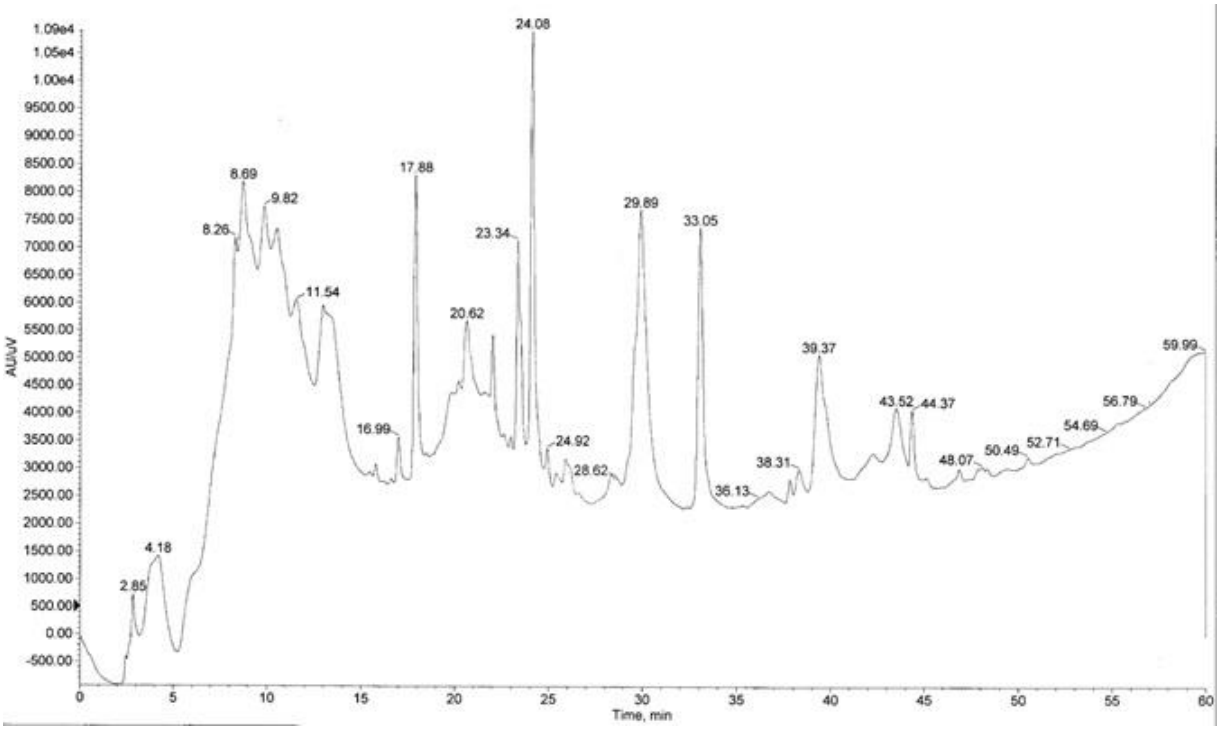
Appendix 75. HPLC-MS chromatogram of allinase reaction on S-(2-pyrrolyl)cysteine at 26.91 min after 2h



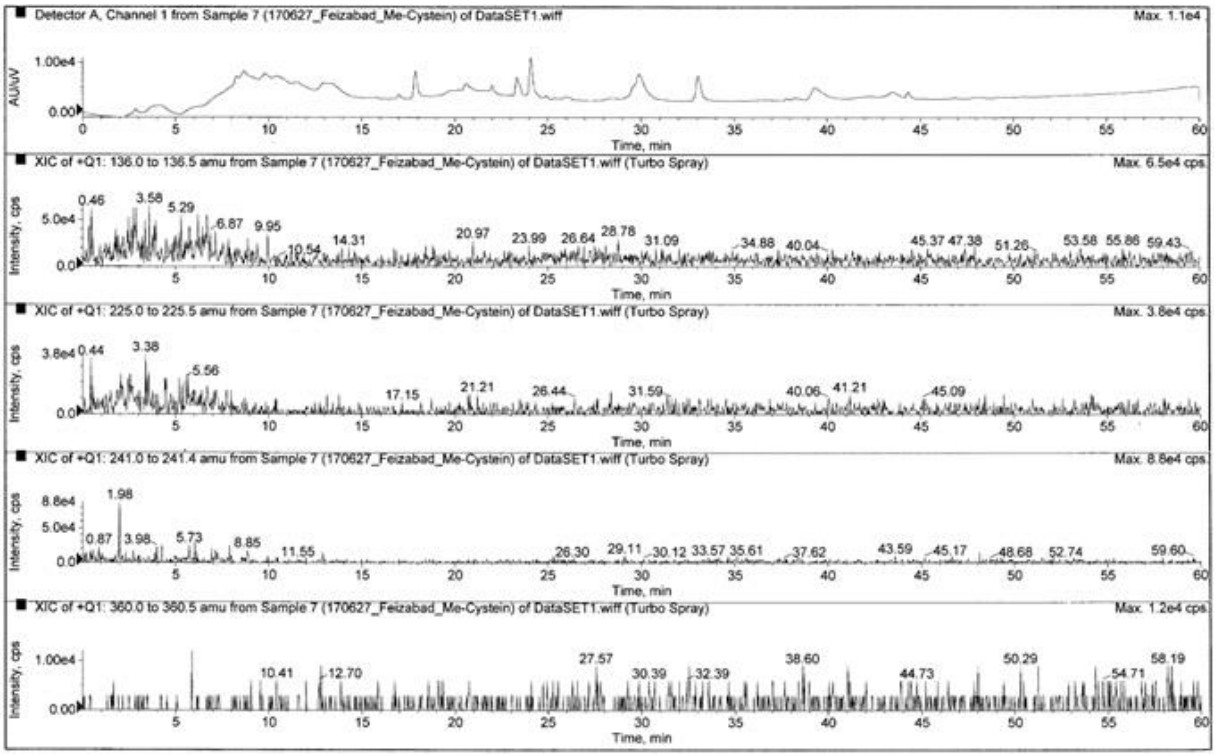
Appendix 76. HPLC-MS chromatogram of allinase reaction on S-(2-pyrrolyl)cysteine at 32.87 min after 2h.



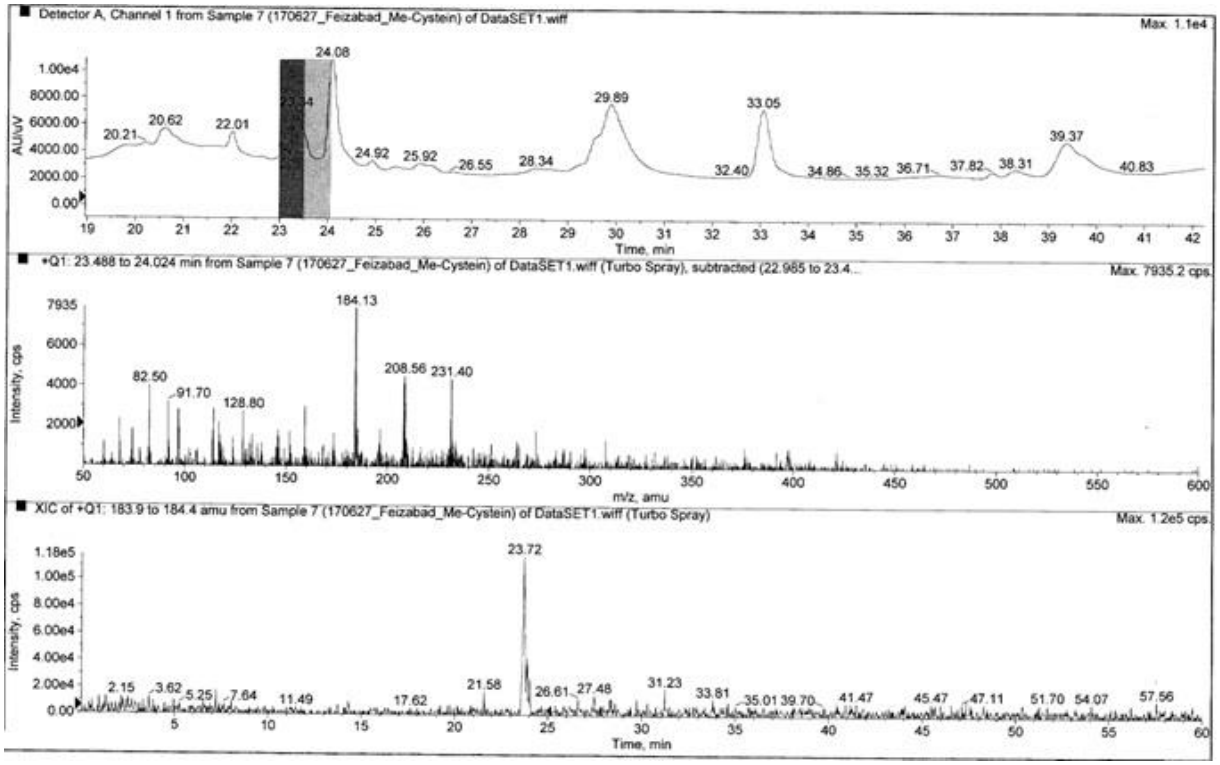
Appendix 77. HPLC chromatogram of allinase reaction on S-(N-methylpyrrol-2-yl)cysteine after 2h.



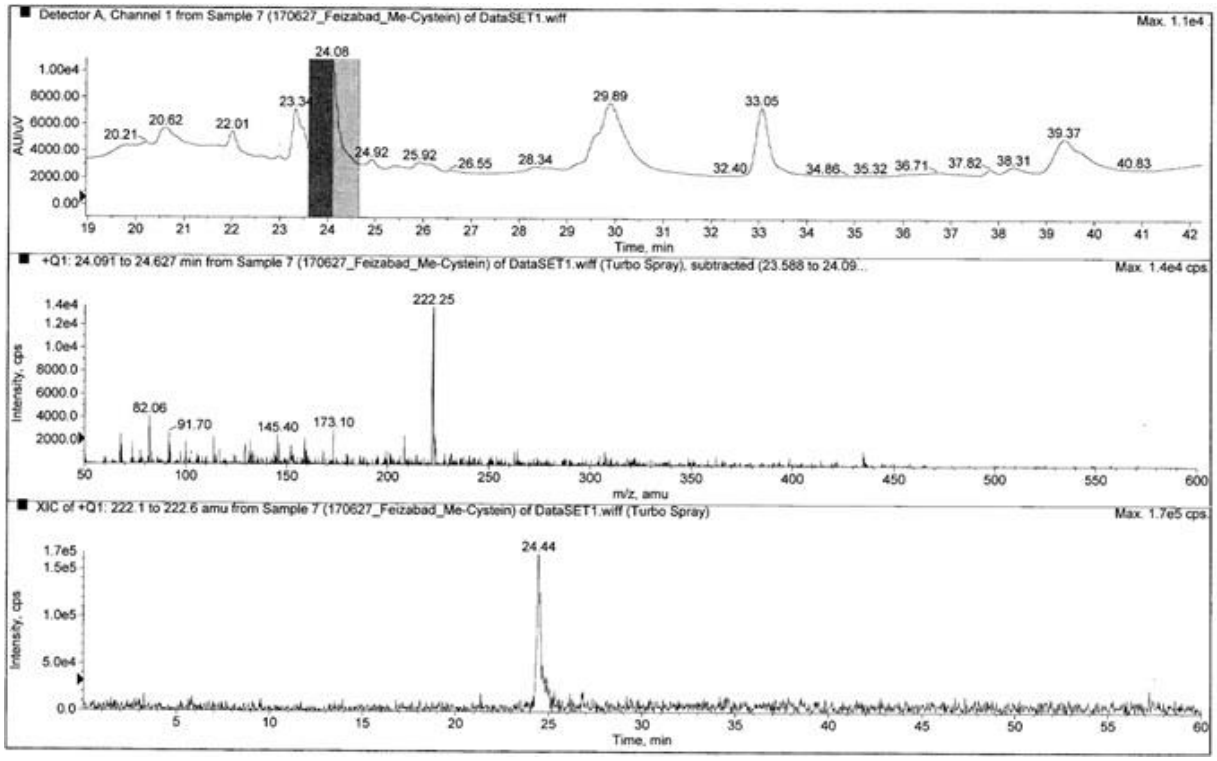
Appendix 78. HPLC-MS chromatogram of allinase reaction on S-(N-methylpyrrol-2-yl)cysteine after 2h.



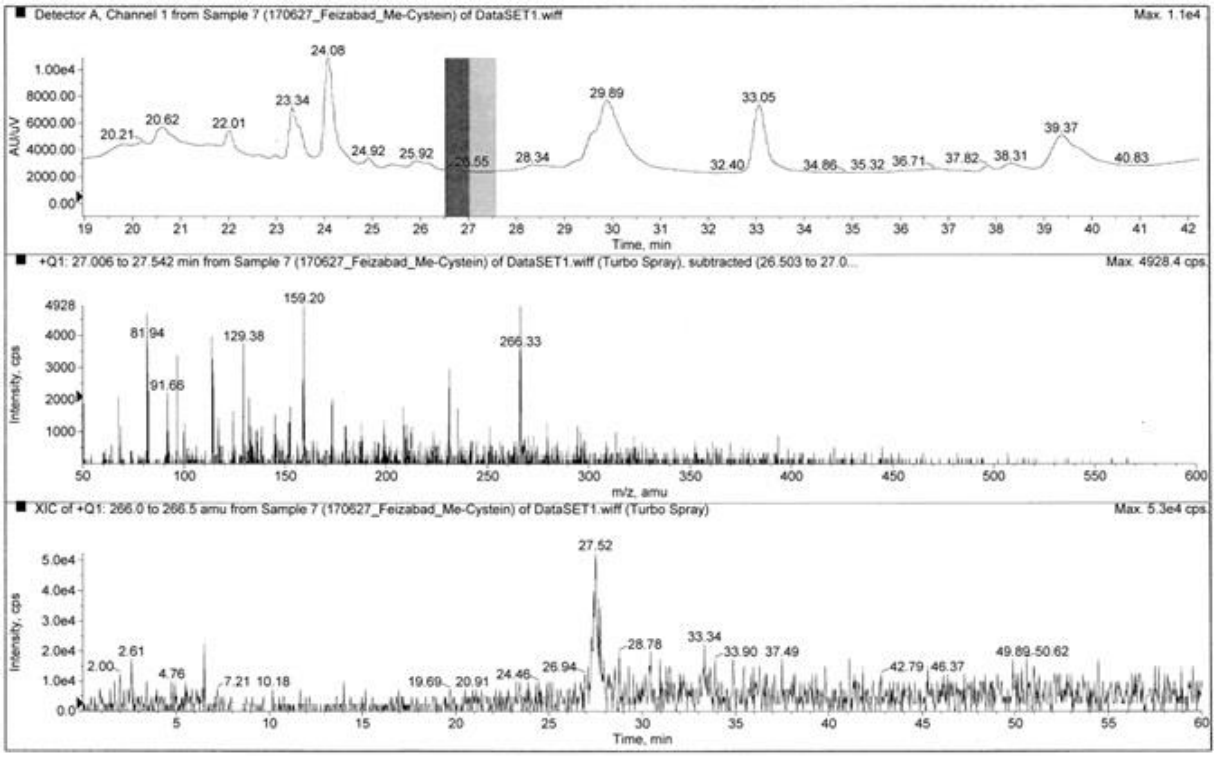
Appendix 79. HPLC-MS chromatogram of allinase reaction onS-(N-methylpyrrol-2-yl)cysteine at 23.72 min after 2h.



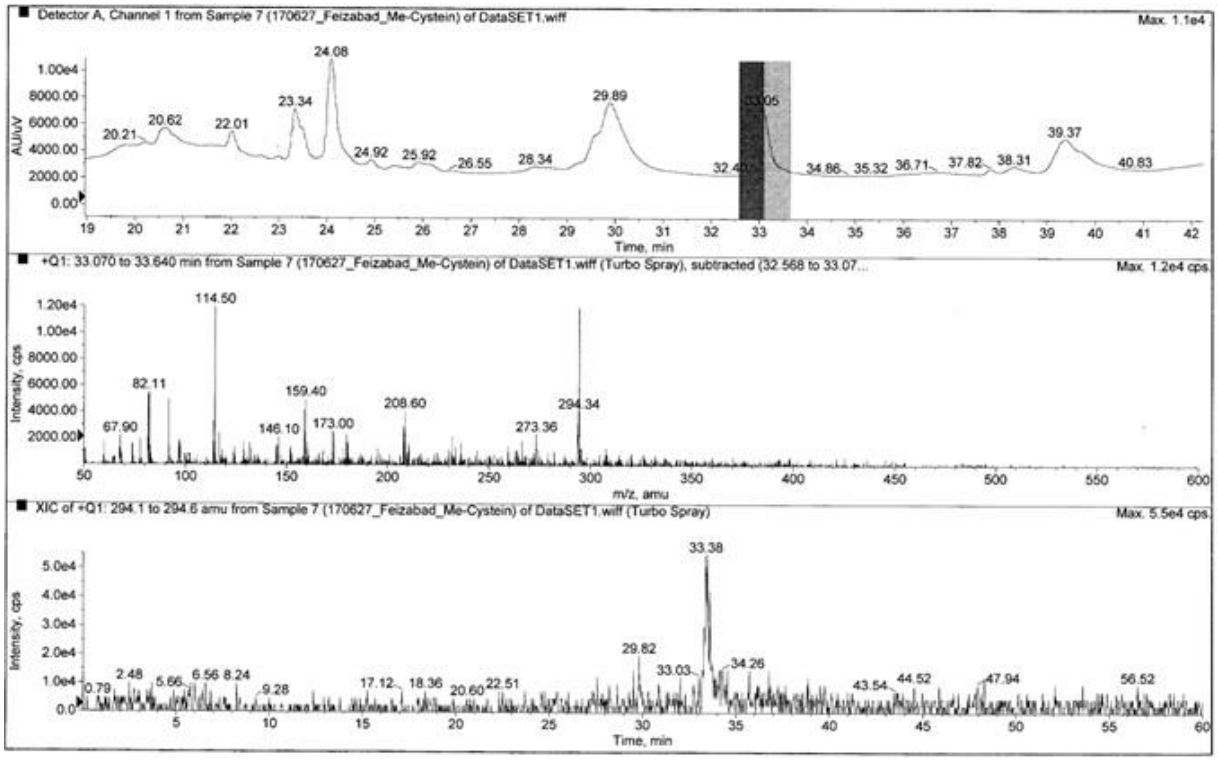
Appendix 80. HPLC-MS chromatogram of allinase reaction on S-(N-methylpyrrol-2-yl)cysteine at 24.44 min after 2h.



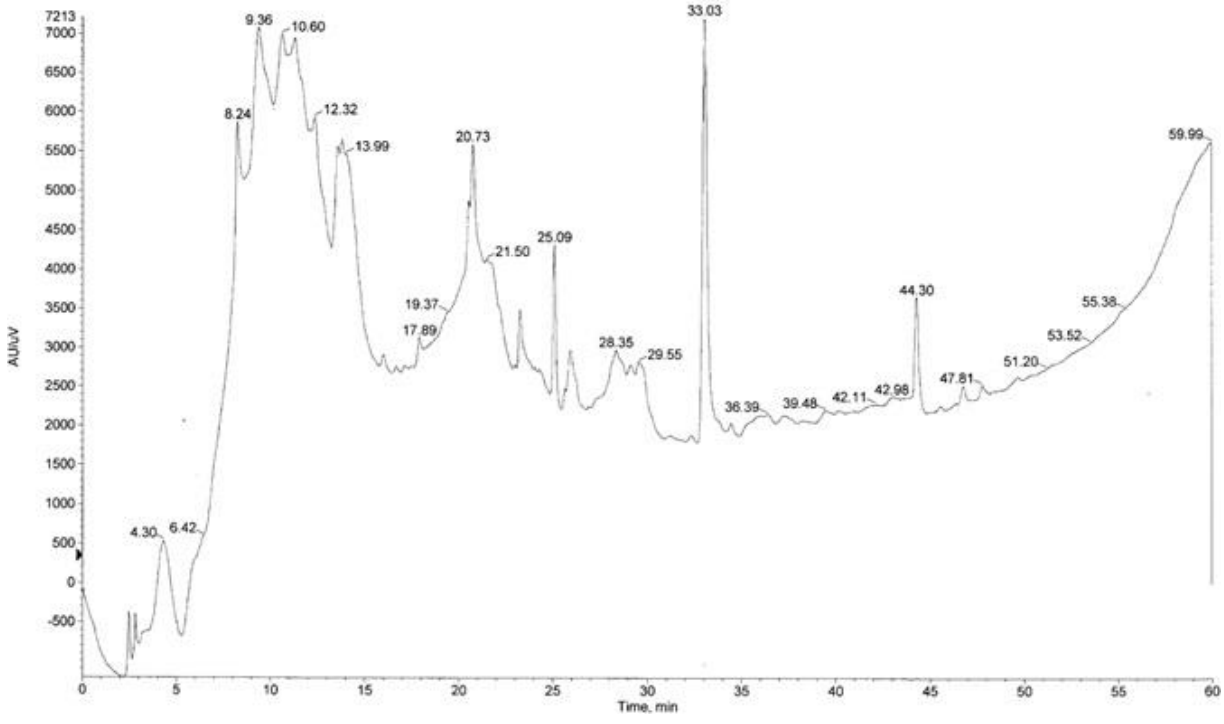
Appendix 81. HPLC-MS chromatogram of allinase reaction on S-(N-methylpyrrol-2-yl)cysteine at 27.52 min after 2h.



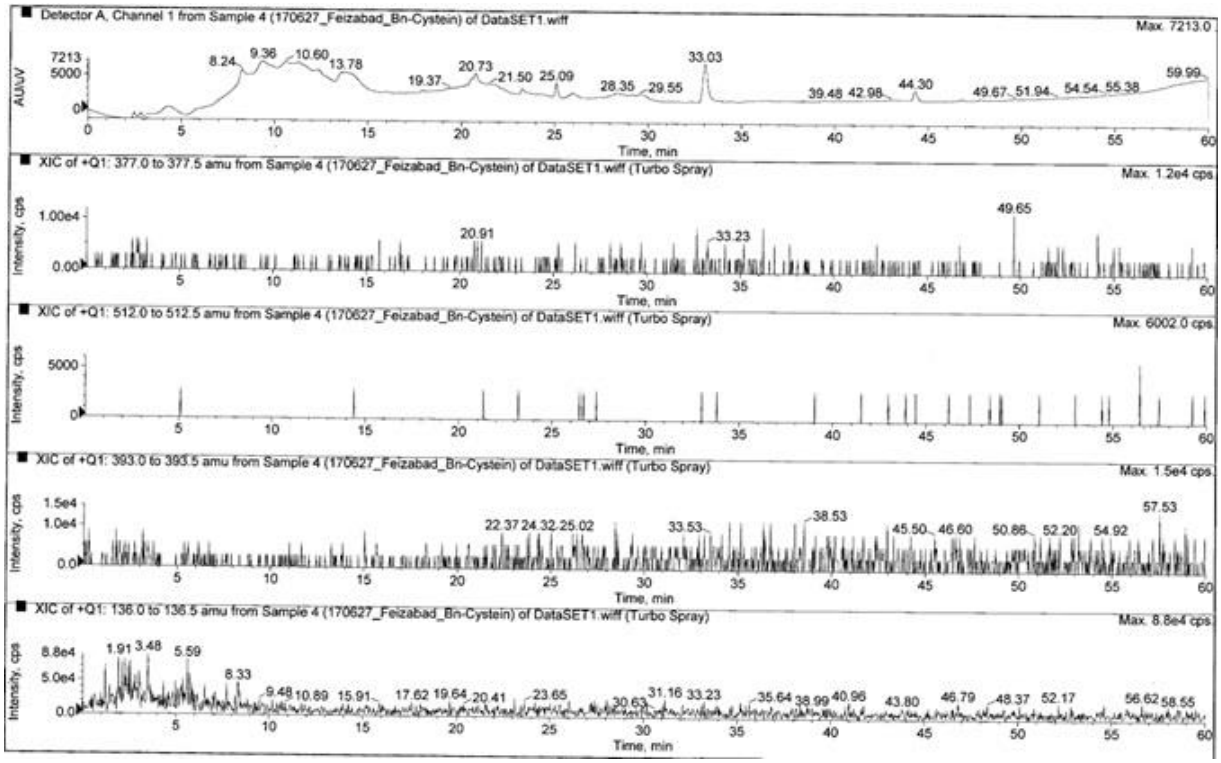
Appendix 82. HPLC-MS chromatogram of allinase reaction on S-(N-methylpyrrol-2-yl)cysteine at 33.38 min after 2h.



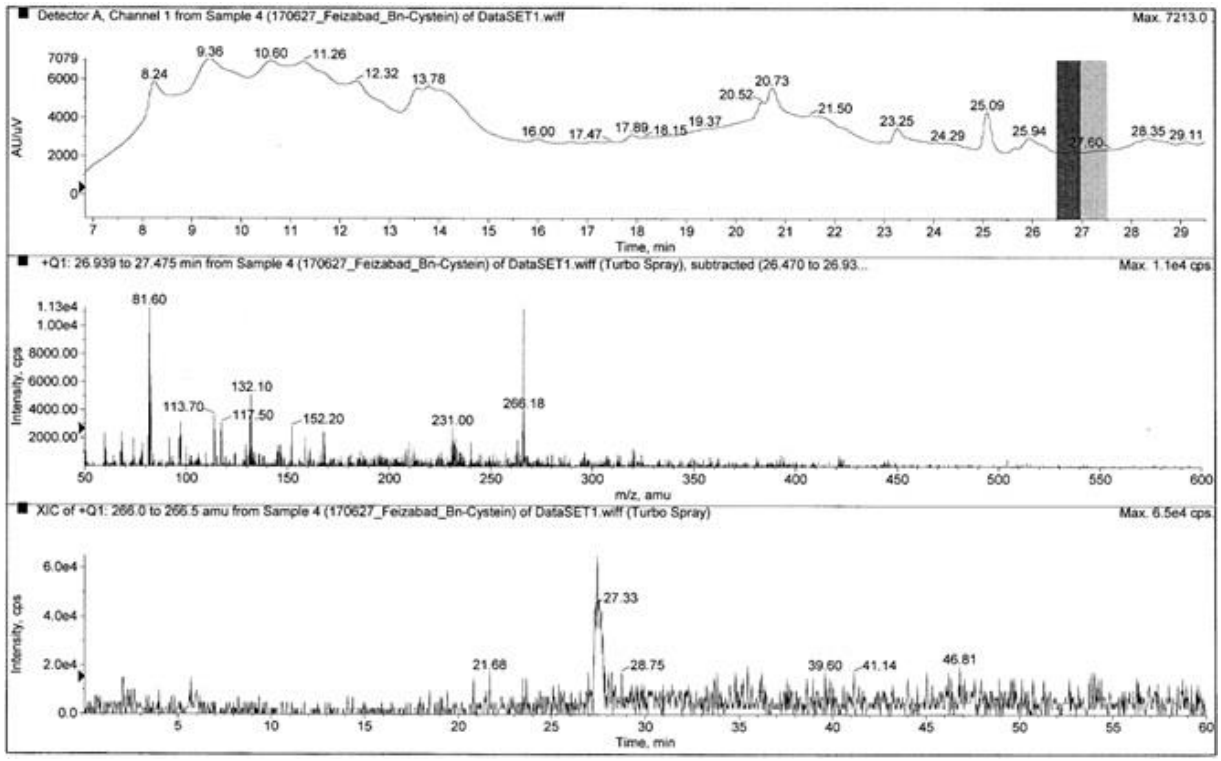
Appendix 83. HPLC chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine after 2h.



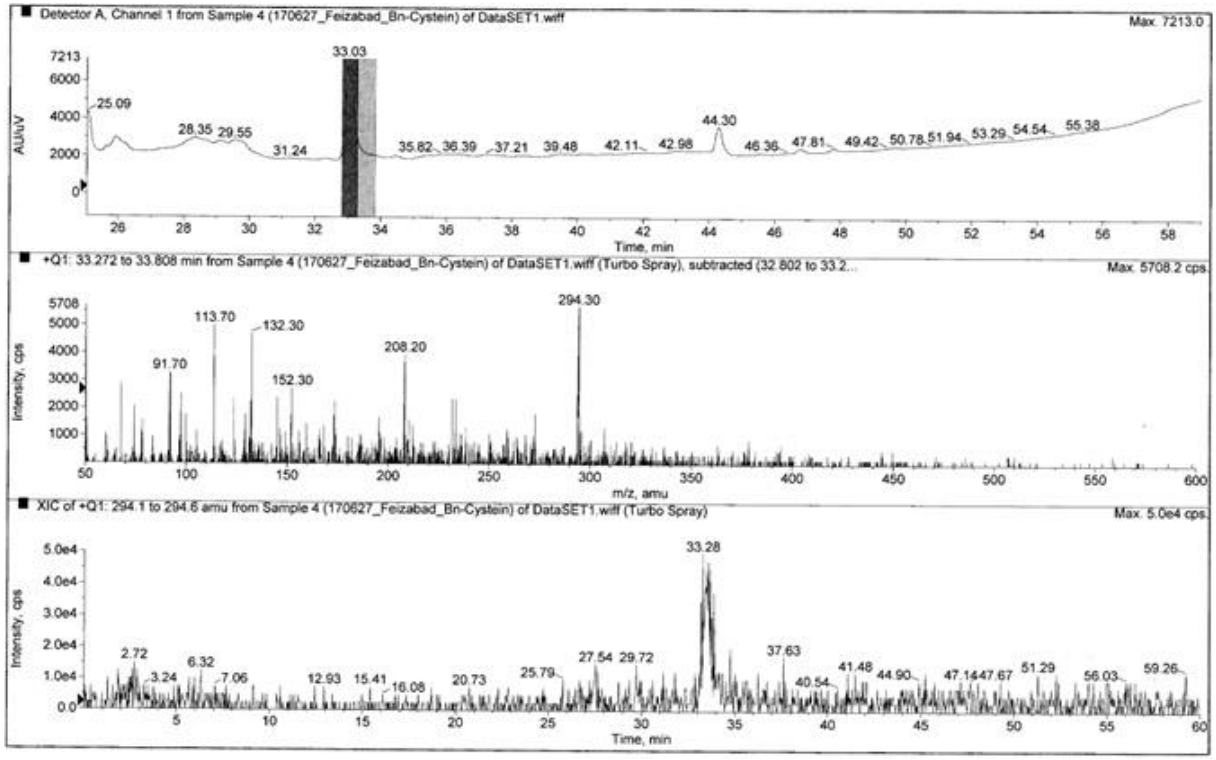
Appendix 84. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine after 2h.



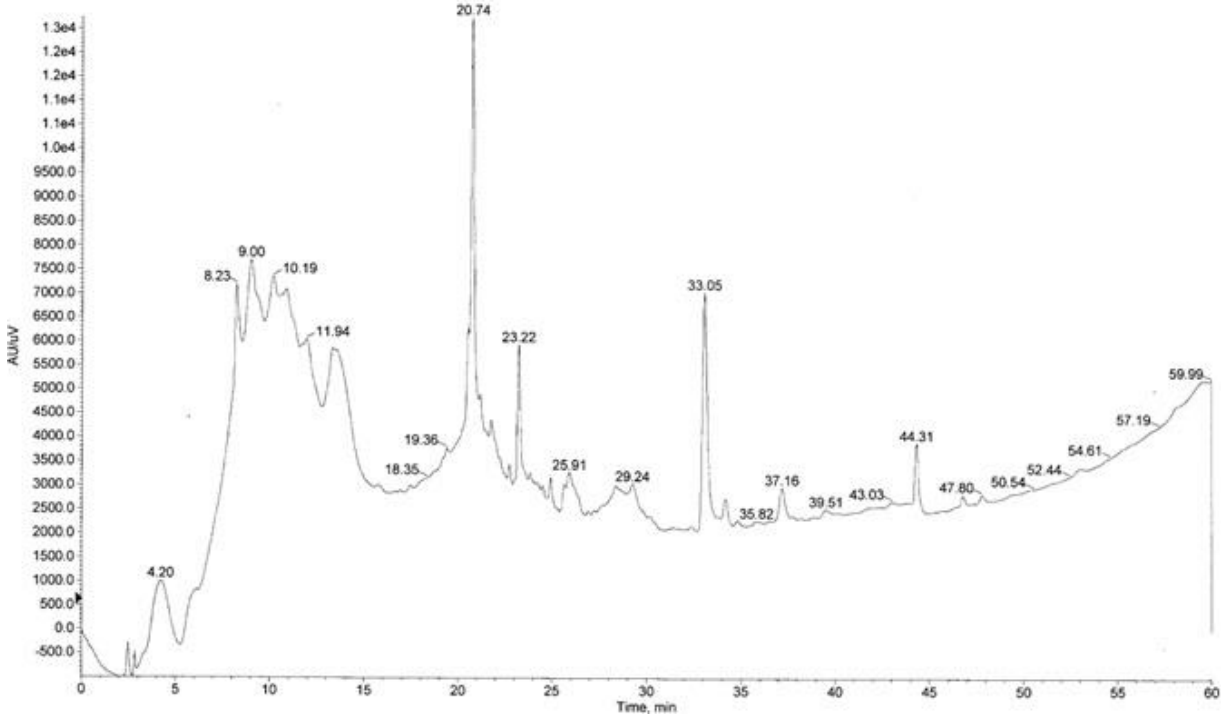
Appendix 85. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine at 27.33 min after 2h.



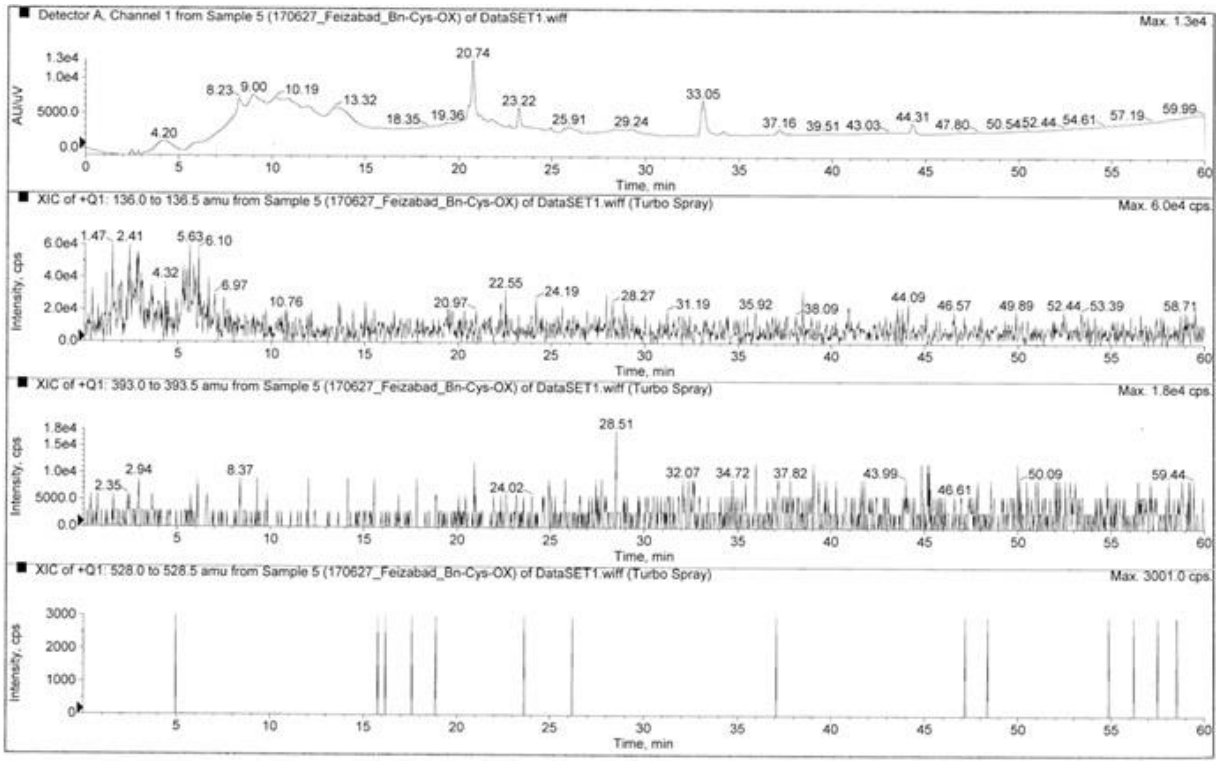
Appendix 86. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine at 33.28 min after 2h.



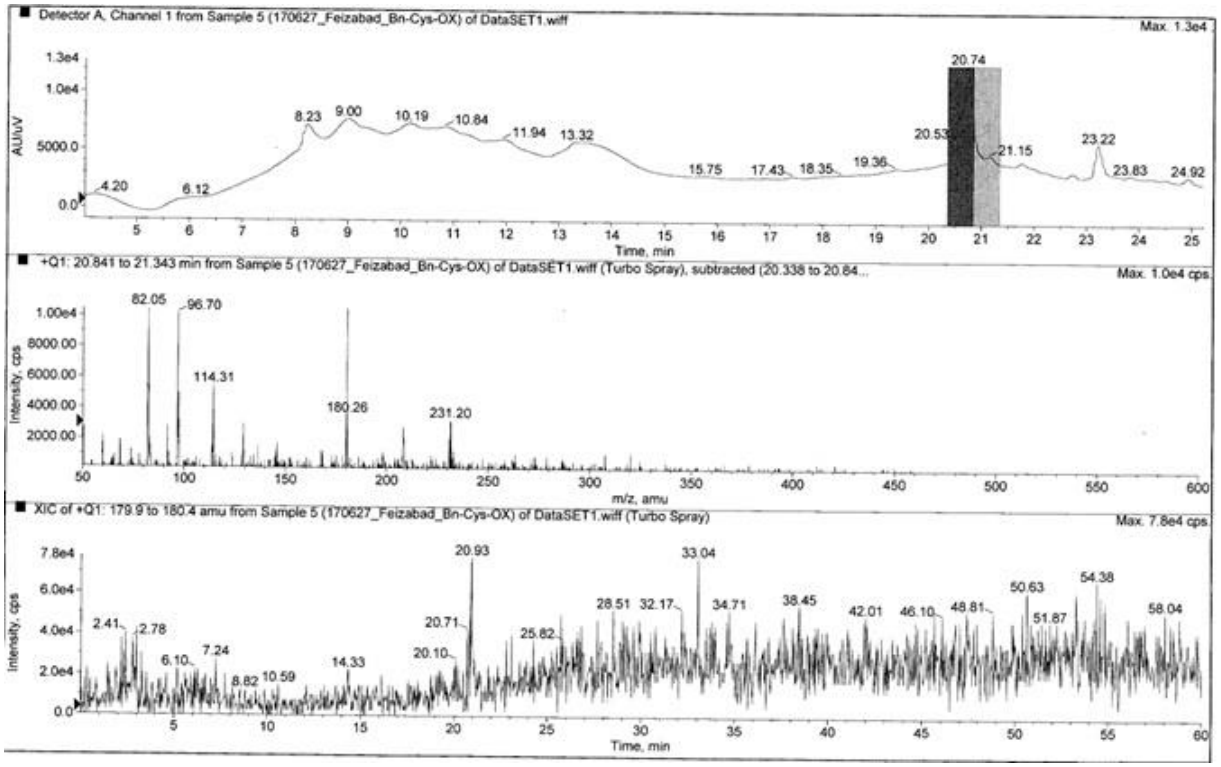
Appendix 87. HPLC chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine-S-oxide after 2h.



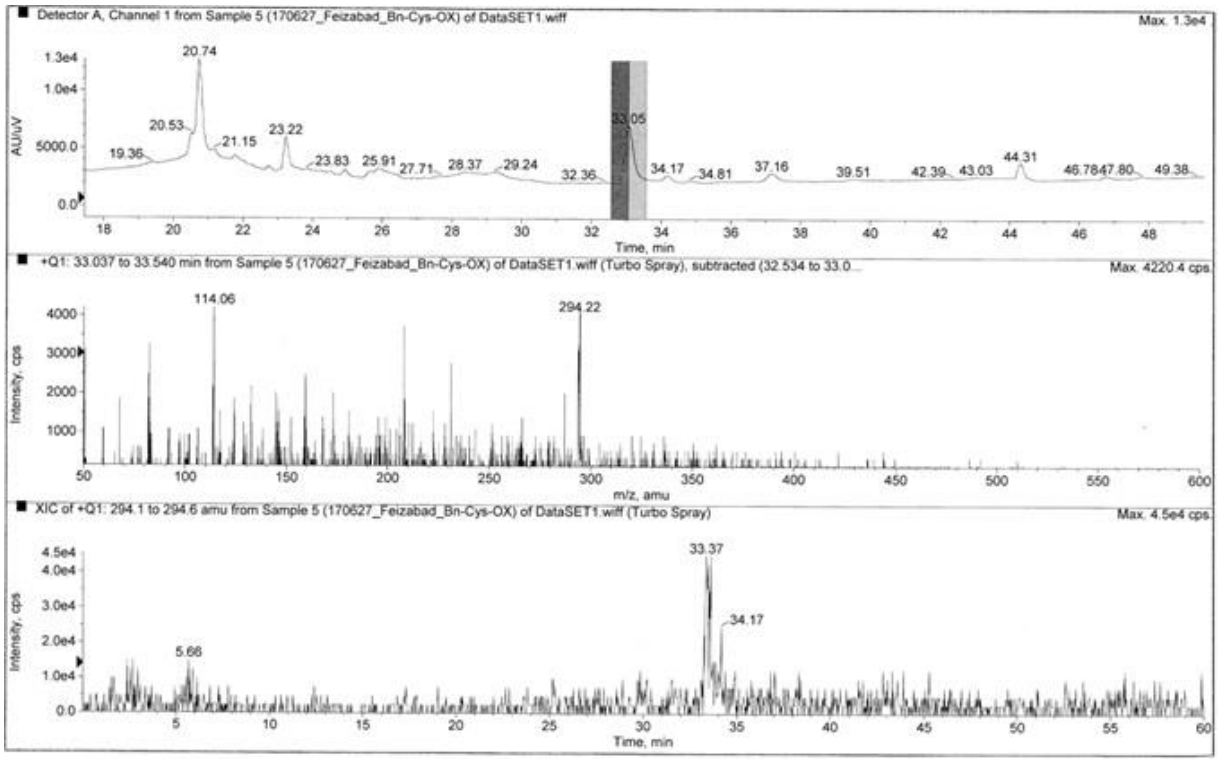
Appendix 88. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine-S-oxide after 2h.



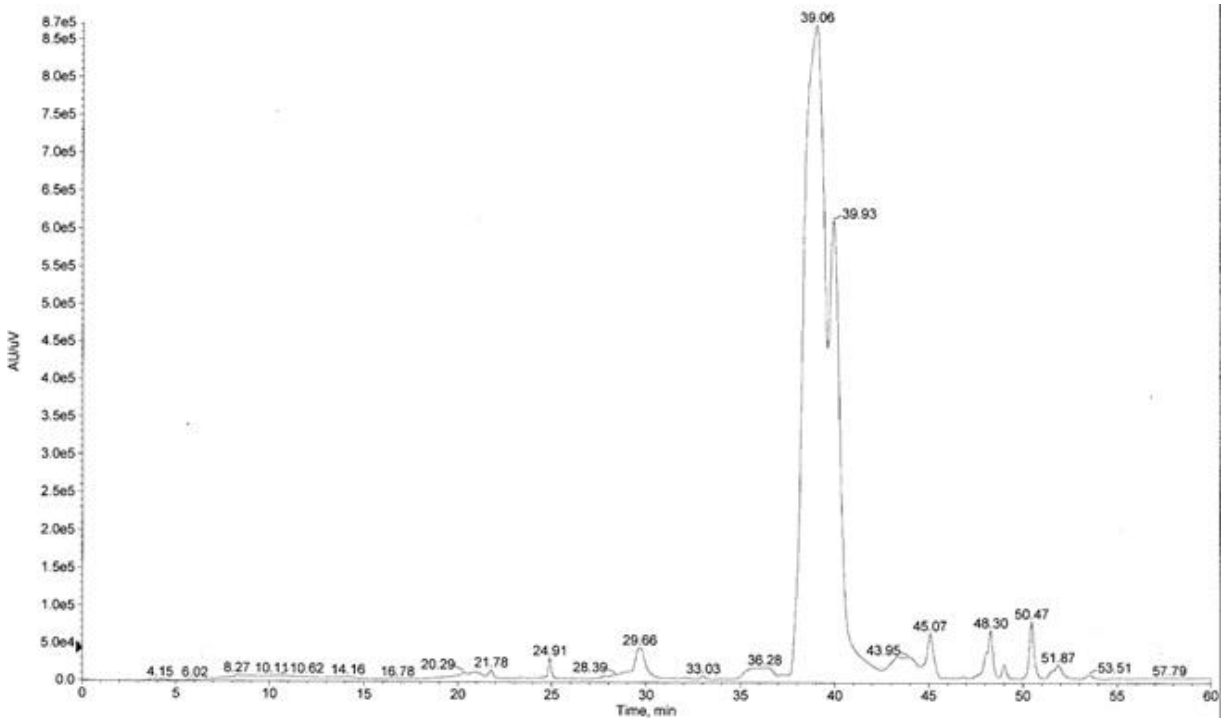
Appendix 89. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine-S-oxide at 20.93 min after 2h.



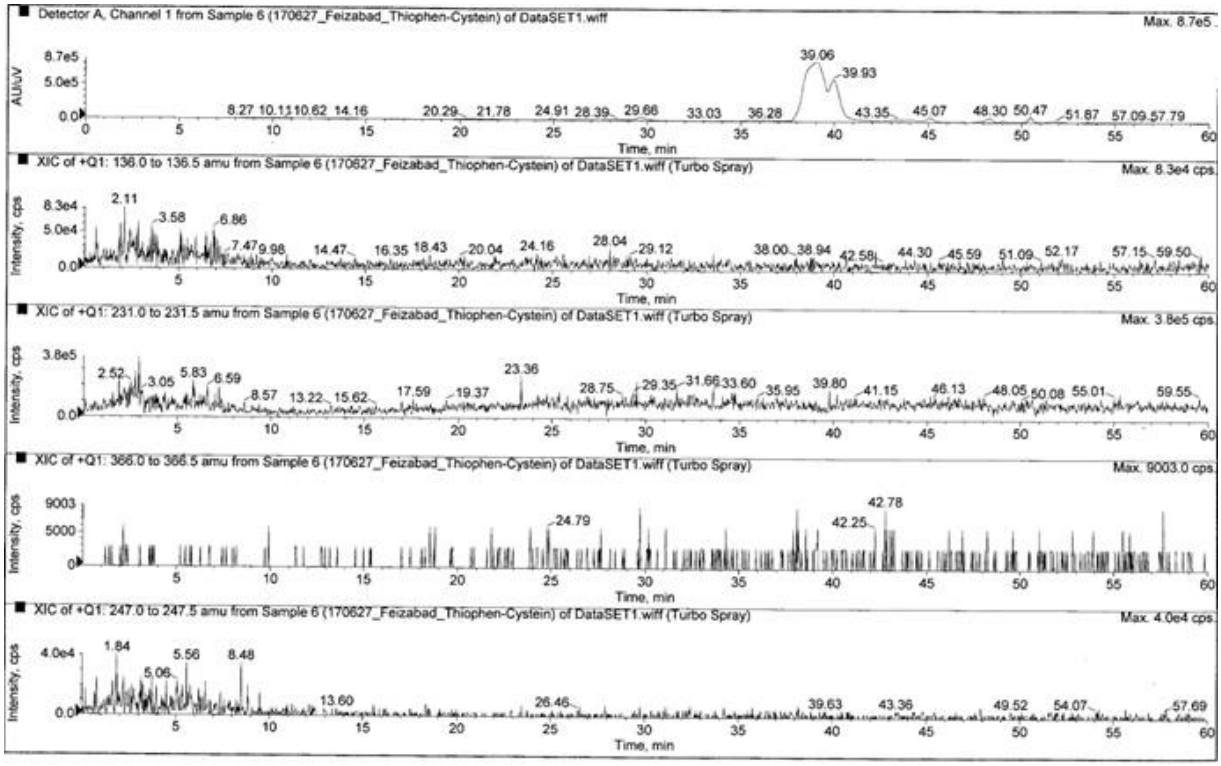
Appendix 90. HPLC-MS chromatogram of allinase reaction on S-(N-benzylpyrrol-2-yl)cysteine-S-oxide at 33.37 min after 2h.



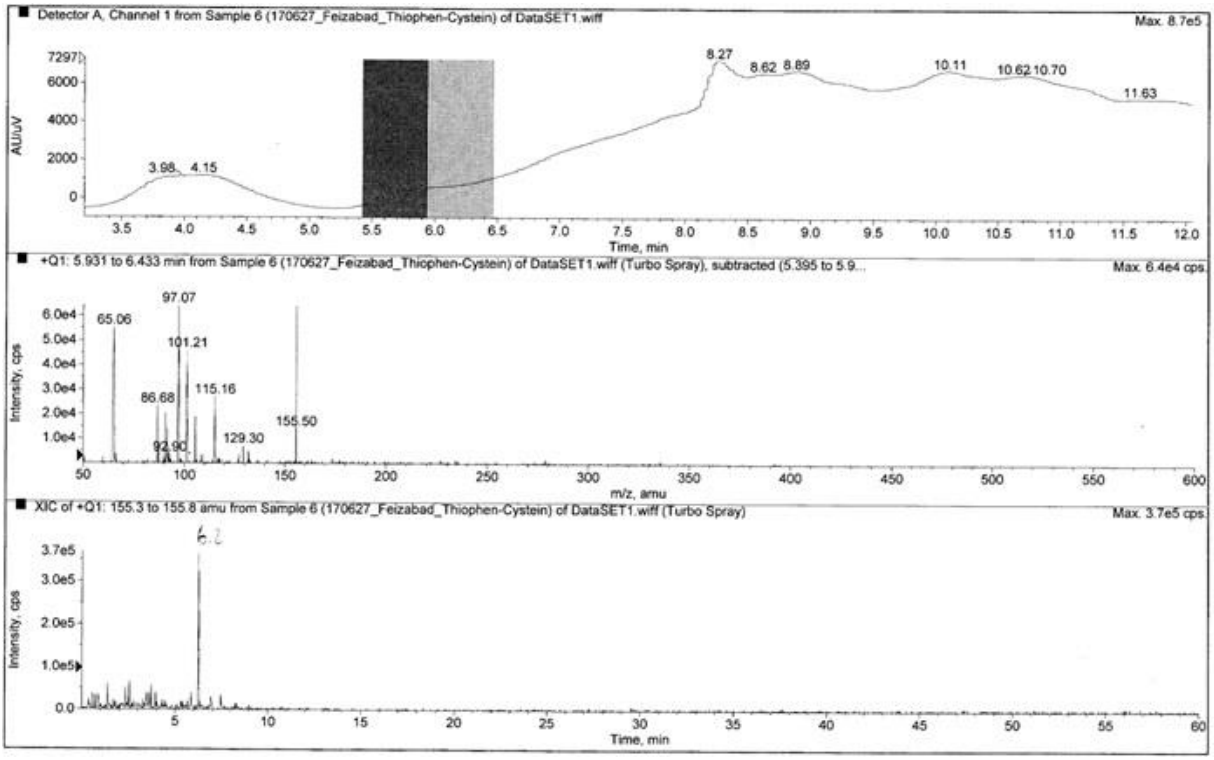
Appendix 91. HPLC chromatogram of allinase reaction on S-(2-thienyl)cysteine after 2h.



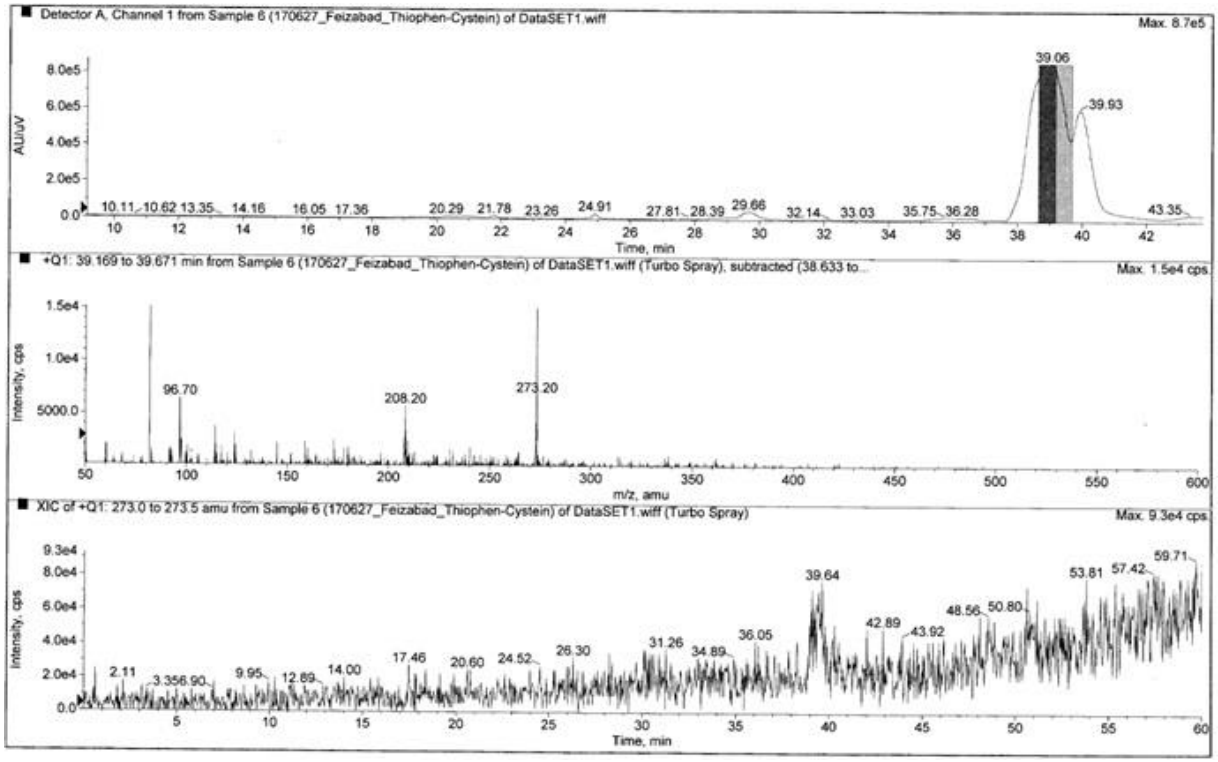
Appendix 92. HPLC-MS chromatogram of allinase reaction on S-(2-thienyl)cysteine after 2h.



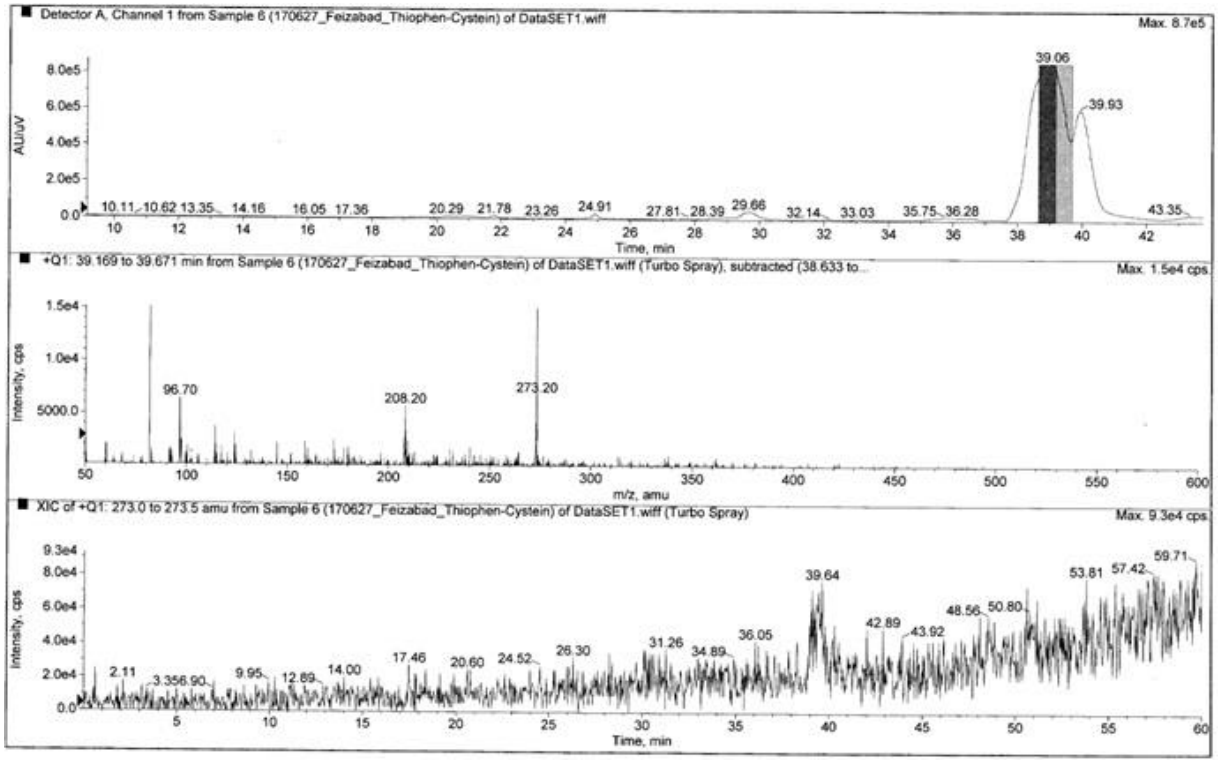
Appendix 93. HPLC-MS chromatogram of allinase reaction on S-(2-thienyl)cysteine at 6.2 min after 2h.



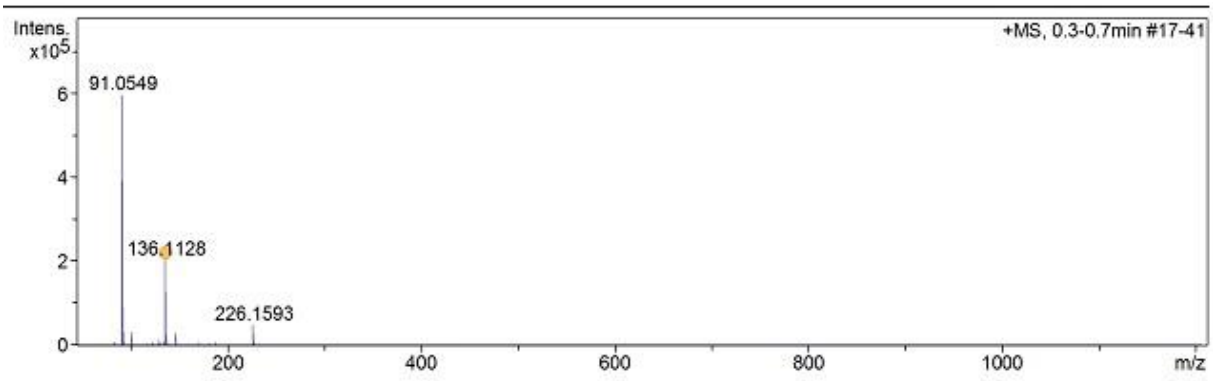
Appendix 94. HPLC-MS chromatogram of allinase reaction on S-(2-thienyl)cysteine at 39.64 min after 2h.



Appendix 95. HPLC-MS chromatogram of allinase reaction on S-(2-thienyl)cysteine at 54.38 min after 2h.

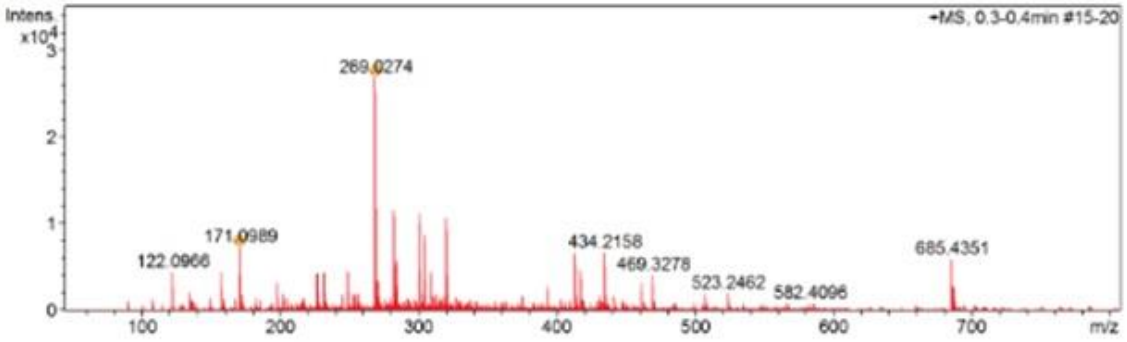


Appendix 96. HR-MS analysis of compound m/z 136 $[M+H]^+$.



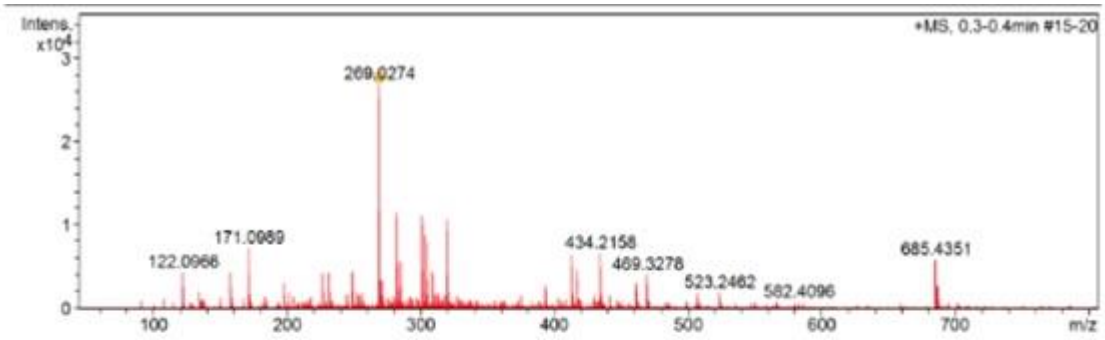
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
136.1128	1	C9H14N	136.1121	-5.4	2.5	1	100.00	3.5	even	ok

Appendix 97. HR-MS analysis of compound m/z 171 $[M+H]^+$



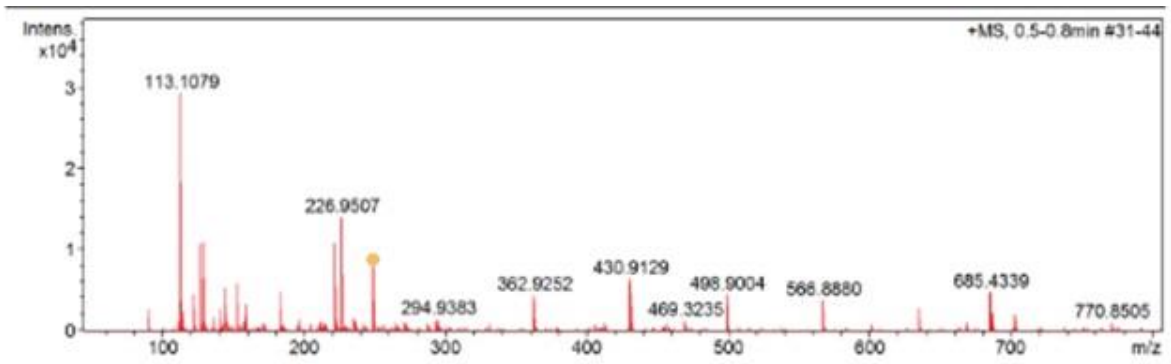
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
171.0989	1	C10H13F2	171.0990	-5.3	10.3	1	100.00	3.5	even	ok
	2	C9H15O3	171.1016	15.7	16.5	2	24.42	2.5	even	ok
	3	C7H14F3O	171.0991	1.4	26.4	3	94.55	-0.5	even	ok
	4	C6H15N2S	171.0950	-22.4	31.6	4	5.31	2.5	even	ok

Appendix 98. HR-MS analysis of compound m/z 269 $[M+H]^+$



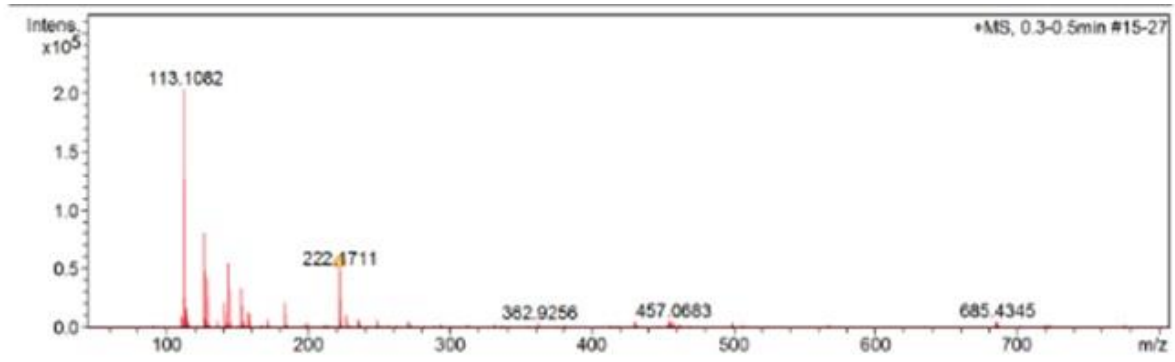
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
269.0274	1	C10H4F3N4O2	269.0251	2.4	7.4	1	60.70	9.5	even	ok
	2	C6HN10O2	269.0276	1.5	7.5	2	66.17	13.5	even	ok
	3	C6H9N6OS2	269.0274	-0.2	10.3	3	100.00	7.5	even	ok
	4	C10H12F3OS2	269.0276	0.7	10.3	4	95.49	3.5	even	ok
	5	C9H14FO4S2	269.0312	14.0	10.7	5	6.12	2.5	even	ok
	6	C12H7F2O5	269.0256	-6.6	10.7	6	27.42	8.5	even	ok
	7	C12H13O3S2	269.0301	9.7	12.3	7	16.81	6.5	even	ok
	8	C12H5N4O4	269.0305	11.5	14.1	8	8.79	12.5	even	ok
	9	C11H9O6	269.0292	6.5	14.1	9	27.30	7.5	even	ok
	10	C10H15F2S3	269.0296	8.9	14.7	10	27.06	2.5	even	ok
	11	C8H14FN2OS3	269.0247	-10.3	15.2	11	19.54	2.5	even	ok
	12	C7H13N2O5S2	269.0260	-5.2	15.9	12	44.51	2.5	even	ok
	13	C13H3F2N4O	269.0269	-1.9	16.7	13	54.59	13.5	even	ok
	14	C6F3N10	269.0254	-7.6	16.9	14	20.79	10.5	even	ok
	15	C13H11F2S2	269.0265	-3.6	17.0	15	56.65	7.5	even	ok
	16	C11H13N2S3	269.0235	-14.5	18.7	16	5.53	6.5	even	ok
	17	C9H5F3O6	269.0267	-2.6	19.6	17	46.45	4.5	even	ok
	18	C7H5N6O6	269.0265	-3.5	19.8	18	40.76	8.5	even	ok
	19	C15H6FO4	269.0245	-11.1	23.1	19	8.06	12.5	even	ok
	20	C8H10FO9	269.0303	10.8	26.0	20	8.21	3.5	even	ok
	21	C12H10FO4S	269.0276	1.5	27.5	21	60.40	7.5	even	ok
	22	C10H7F2N4OS	269.0303	10.7	30.6	22	10.03	8.5	even	ok
	23	C11H5N6OS	269.0240	-12.6	30.9	23	5.64	12.5	even	ok
	24	C13H5F3OS	269.0242	-11.9	31.0	24	7.23	8.5	even	ok
	25	C15H9O3S	269.0267	-2.6	33.3	25	43.92	11.5	even	ok
	26	C6H9N2O10	269.0252	-8.4	33.6	26	11.63	3.5	even	ok
	27	C13H6FN4S	269.0292	6.4	33.8	27	23.72	12.5	even	ok
	28	C9H11F2O5S	269.0290	5.7	34.3	28	20.07	3.5	even	ok
	29	C16H2FN4	269.0256	-6.1	34.6	29	16.53	17.5	even	ok
	30	C8H6FN6O2S	269.0251	-8.5	35.6	30	11.03	8.5	even	ok
	31	C7H10FN2O6S	269.0236	-13.5	37.7	31	2.66	3.5	even	ok
	32	C7H6F3N4O2S	269.0315	14.9	40.0	32	1.74	4.5	even	ok
	33	C6H12F3O6S	269.0301	10.0	44.9	33	6.15	-0.5	even	ok

Appendix 99. HR-MS analysis of compound m/z 249 $[M+H]^+$



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
249.1094	1	C10H13N6O2	249.1095	0.0	4.7	1	100.00	7.5	even	ok
	2	C14H17O4	249.1121	10.8	11.1	2	16.01	6.5	even	ok
	3	C9H17N2O6	249.1051	-5.4	15.6	3	41.21	2.5	even	ok
	4	C13H17N2OS	249.1056	-15.4	22.7	4	5.15	6.5	even	ok
	5	C15H13N4	249.1135	16.2	24.7	5	2.97	11.5	even	ok
	6	C7H17N6O2S	249.1126	13.5	25.5	6	6.06	2.5	even	ok
	7	C10H21N2OS2	249.1090	-1.9	40.5	7	49.13	1.5	even	ok

Appendix 100. HR-MS analysis of compound m/z 222 $[M+H]^+$



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
222.1711	1	C11H20N5	222.1713	0.6	4.1	1	100.00	4.5	even	ok
	2	C10H24NO4	222.1700	-5.2	10.5	2	54.09	-0.5	even	ok
	3	C6H24N5S	222.1747	16.0	31.3	3	4.64	-0.5	even	ok